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ScienceDirect
Theriogenology xxx (2008) xxx–xxx
Theriogenology
www.theriojournal.com

Low peripheral progesterone and late embryonic/early fetal loss in suckled beef and lactating dairy cows☆

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Received 14 February 2008; received in revised form 14 July 2008; accepted 26 July 2008

Abstract

Pregnancy failure during placentation in lactating dairy cows was associated with low concentrations of serum progesterone. Beef cows have greater serum progesterone and less pregnancy failure. Experiment 1 determined that reduction of serum progesterone affected late embryonic/early fetal loss in suckled beef cows. Cows ($n = 40$) received progesterone from two new or used controlled internal drug releasing devices, replaced every 5 d, beginning on Day 28 of gestation (mating = Day 0); CL were enucleated on Day 29. Retention of pregnancy was 77% in treated cows and 97% in 78 control cows ($P < 0.05$). Experiment 2 determined how pregnant, lactating dairy cows with high or low progesterone concentrations during Days 28–34 differed in luteal function or in serum progesterone during replacement therapy. Luteal tissue from such cows was assayed for progesterone and expression of mRNA for genes of endothelin and prostaglandin (PG) systems. Secretion of progesterone and prostaglandins by dispersed luteal cells was determined during incubation with LH, endothelin-1, or arachidonic acid. Neither luteal progesterone nor mRNAs for endothelin or prostaglandin systems differed. Endothelin-1 inhibited secretion of progesterone more ($P < 0.05$) in luteal cells from cows with low versus high serum progesterone, when incubated with arachidonic acid. Secretion of prostaglandin $F_2\alpha$ was increased and that of 6-keto-$PGF_1\alpha$ decreased by endothelin-1 *in vitro*. Serum progesterone during replacement was lower ($P < 0.05$) for cows with low than high serum progesterone at lutectomy. Thus, clearance, more than luteal production, determined peripheral progesterone in pregnant, lactating dairy cows.

Keywords: Embryonic mortality; Progesterone; Pregnancy; Beef cows; Dairy cows

1. Introduction

Failure of lactating dairy cows to establish and maintain pregnancy is a substantial impediment to production efficiency [1]. During late embryonic/early fetal development (Days 30–60 post-insemination) formation of an efficient system for transfer of nutrients and wastes between dam and fetus is required to support fetal growth [2]. Poor placentation has been suggested as the leading cause of late embryonic losses between

☆ This work was supported by NRI Competitive Grant # 2002-35203-12230 from the USDA Cooperative State Research, Education, and Extension Service and Hatch Project 427 (NE 1007) of the West Virginia Agricultural and Forestry Experiment Station. It is published with the approval of the Director of the Station as Scientific Paper No. 3014.

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doi:10.1016/j.theriogenology.2008.07.031

Days 30 and 45 of gestation [3]. Pregnancies were lost between Days 28 and 98 of gestation in 7–33% of lactating dairy cows [1,4]. Most pregnancy loss after Day 30 in dairy cows and heifers had occurred by Day 42 [5], and losses increased as circulating concentrations of progesterone around Day 30 of pregnancy decreased.

Peripheral progesterone concentrations are a net result of secretion and metabolism. Increased metabolism by the liver has been hypothesized to lower serum concentrations of progesterone during the estrous cycle in lactating dairy cows [6]. Liver blood flow and metabolic clearance rates for progesterone and estradiol-17β were increased acutely in lactating and non-lactating dairy cows fed above maintenance [7]. Greater feed intake reduced serum progesterone during the luteal phase or during treatment with exogenous progesterone [8–11].

Alternatively, low concentrations of progesterone could be due to enhanced luteolytic or anti-luteotropic actions within the CL. Studies in CL of non-pregnant cows and ewes have indicated that the endothelin system is important during prostaglandin (PG) $F_2\alpha$-induced regression [12–15]. Bovine CL synthesize endothelin-1 (EDN1) [13], and both the mRNA and EDN1 protein varied with stage of estrous cycle and pregnancy status [16]. Expression of preproendothelin-1 (*EDN1*) was stimulated by $PGF_2\alpha$ *in vitro* and *in vivo* during the midluteal phase [14,17]. Although exact roles remain to be elucidated [12,18,19], both $PGF_2\alpha$ and EDN1 appear to be involved in functional luteolysis in non-pregnant cows and ewes [15].

Although uterine $PGF_2\alpha$ initiates luteolysis, CL produce substantial amounts of $PGF_2\alpha$ [20]. Luteal secretion of $PGF_2\alpha$ was reduced by pregnancy in pigs [21,22] and increased by exogenous $PGF_2\alpha$ in ewes and pigs [23,24]. In the cow, synthesis of PGI_2 (measured as its metabolite 6-keto-$PGF_1\alpha$) and $PGF_2\alpha$ by luteal cells *in vitro* varied with stage of the estrous cycle [25]. Tsai and Wiltbank [26,27] proposed that secretion of $PGF_2\alpha$ from the CL amplified the luteolytic signal from the uterus. A single injection of $PGF_2\alpha$ upregulated mRNA encoding prostaglandin G/H synthase 2 (PGHS-2, COX-2) in mid- and late-cycle ovine and bovine CL, but only repeated injections were effective in animals on Day 4 of the estrous cycle [28]. Arosh et al. [29] suggested that increased luteal prostaglandins mediate luteolysis, because enzymatic activity has shifted to favor synthesis of $PGF_2\alpha$ rather than PGE_2.

Pregnancy failure during placentation is less frequent in beef cattle. Only 2–6% of pregnancies were lost during late embryonic/early fetal development in beef cows [4]. Lactating dairy cows had lower concentrations of serum progesterone than beef cows [7,30,31]. It has not been determined for beef cows if reduced concentrations of progesterone affect retention of pregnancy. Therefore, Experiment 1 was designed to examine retention of pregnancy in suckled beef cows maintained on lower than normal concentrations of progesterone from Days 29 through 53 of gestation.

Whether low circulating concentrations of progesterone in pregnant, lactating dairy cows are the result of decreased luteal production, increased clearance, or both, has not been determined. Experiment 2 was designed to compare: (1) progesterone content and secretion *in vitro*, (2) expression of enzymes involved in synthesis and degradation of prostaglandins $F_2\alpha$ and I_2, and (3) expression of components of the luteal endothelin system, in CL collected from lactating dairy cows with low or high peripheral progesterone on Day 30 of pregnancy and to compare concentrations of progesterone in peripheral blood of these cows when a standard dosage of progesterone was injected after lutectomy.

2. Materials and methods

All procedures for each experiment were approved by the Animal Care and Use Committee of West Virginia University (ACUC # 03-0402 and 03-0502).

2.1. Experiment 1

Forty suckled beef cows of mixed breeds were assigned randomly within age class (2–3 years, $n = 22$; $\geq$4 years, $n = 18$) and uterine horn of pregnancy (right or left) to two treatment groups. The cows were pregnant from inseminations at 40–65 d postpartum. Remaining pregnant cows in the herd ($n = 78$) served as an untreated reference group. Treated cows received either two new (1.38 g progesterone per insert; $n = 19$) or two used (for 7 d, then cleaned with warm, soapy water; $n = 21$) controlled internal drug releasing devices (CIDR; Pfizer Animal Health, New York, NY, USA) on Day 28 after first-service AI. The rationale was that two new CIDR inserts should deliver enough progesterone to maintain pregnancy [32]. The CL of pregnancy was removed by transrectal enucleation on Day 29. The CIDR inserts were replaced every 5 d from Day 33 until Day 53 or loss of pregnancy. Throughout the study, cows grazed rotationally on pastures that contained primarily a mixture of orchard grass, tall fescue, and red or white dutch clover.

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Retention of pregnancy in the treated cows, based on visualization of an embryonic or fetal heartbeat, was evaluated daily by transrectal ultrasonography (using an Aloka 500 console with a 7.5 MHz linear-array transducer; Corometrics Medical Systems, Wallingford, CT, USA) between Days 28 and 38, and then on Days 40, 43, 45, 48 and 53 or until loss of pregnancy. Days 38 and 53 were chosen arbitrarily as end points near the conclusion of placentation and the transition from embryo to fetus. At each ultrasonographic examination, crown-rump length of the embryo or fetus, and diameters and locations of the two largest follicles ≥5 mm and any CL on each ovary were recorded. Retention of pregnancy in the reference group was evaluated by fall pregnancy examinations in relation to initial ultrasonographic diagnoses at approximately Day 35. A new luteal structure was detected by ultrasonography on or after Day 34 in five experimental cows. This luteal tissue formed after lutectomy and was noted and followed in the records of these cows. Jugular blood samples were collected at each ultrasonographic examination in all 40 experimental cows and on 2 control cows, the latter to indicate normal progesterone concentrations in this herd at this stage of pregnancy. Serum was collected, frozen, and assayed for concentrations of progesterone [33] and estradiol-17β [34,35].

2.2. Experiment 2

Lactating cows in the West Virginia University dairy herd were examined by transrectal ultrasonography (using an Aloka 900 console with a 7.5 MHz linear-array transducer; Corometrics Medical Systems, Wallingford, CT, USA) for the presence of a viable embryo with a heartbeat on Days 28–34 of gestation. Days postpartum averaged 102 ± 8 at initial ultrasonography and jugular blood sampling. The sample was allowed to clot for 6 h, and serum was assayed immediately for progesterone. Only cows with concentrations of progesterone ≤2.5 ng/mL or ≥4.0 ng/mL were selected for the study ($n = 7$ Holstein and 5 Ayrshire). These criteria were chosen to provide a comparison of cows that have an increased risk of pregnancy loss (low progesterone) with cows that would be expected to have a greater pregnancy retention rate (medium to high progesterone) [5]. Milk production on the day of lutectomy averaged 23.3 ± 0.39 kg in the high progesterone group and 23.3 ± 0.16 in the low progesterone group.

Corpora lutea were removed via supravaginal incision as described by Casida [36], under epidural anesthesia with 2% lidocaine hydrochloride, the day after initial sampling for circulating concentration of progesterone. Immediately prior to lutectomy, size of the corpus luteum (two-dimensional diameter at the largest cross-section) and embryonic viability were determined via transrectal ultrasonography. Luteal tissue was placed in cold physiological saline and transported immediately to the laboratory for further processing. A portion of the tissue was placed in cold Medium 199 (M 199; GIBCO, Carlsbad, CA, USA), minced with a sterile scalpel, and shipped, on ice, overnight to the University of Connecticut for studies with dispersed luteal cells. The remaining luteal tissue was cut into small cubes, snap-frozen in liquid nitrogen, and stored at −80 °C.

To obtain an indication of whether metabolism or clearance of progesterone differed between cows with high or low progesterone before lutectomy, jugular concentrations of progesterone were monitored during replacement therapy. Beginning immediately after blood sampling at Hour 0, progesterone (150 mg in 6 mL corn oil; Sigma Chemical Co., St. Louis, MO, USA) was injected subcutaneously every 12 h for the next 2 d. This dosage has been used [37] to maintain pregnancy in cows lutectomized at various times from Days 5 to 35. Jugular blood samples were taken every 4 h beginning at lutectomy (Hour 0) and continuing through Hour 48. Samples were allowed to clot for 12 h, centrifuged at 1500 × *g* for 30 min, and two aliquots of serum were stored at −20 °C.

2.2.1. Real-time RT-PCR

Abundances of mRNA for *EDNs 1* and *3*, endothelin receptors (*EDNR*) *A* and *B*, endothelin converting enzyme-1 (*ECE1*), cyclooxygenase-2 (*COX2*), aldoketoreductase 1B5 (*AKR1B5*), hydroxyprostaglandin dehydrogenase 15 (*HPGD*), and prostaglandin E synthase (*PTGES*) in luteal tissue were measured by quantitative real-time PCR. Total RNA was isolated from frozen luteal tissue using Trizol reagent, according to the manufacturer's instructions (GIBCO BRL, Gaithersburg, MD, USA). The quality of RNA was determined by separation and visualization on a 1.5% agarose gel stained with ethidium bromide and quantified spectrophotometrically at 260 nm. For each sample, 2 μg of DNAse-treated total RNA was reverse transcribed to first-strand cDNA using oligo (dT)18 primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Negative control reverse transcription reactions without the enzyme were carried out to confirm that there was no contamination with genomic DNA.

Real-time PCR primers for the genes listed above and a control gene, glyceraldehyde-3-phosphate dehy-

Table 1
Primer sequences and PCR conditions for examination of luteal tissues

Gene	Primer sequence	Accession number	Product size (bp)	Annealing temperature (°C)	Intra-assay CV (%)[a]
Glyceraldehyde phosphate dehydrogenase (*GAPDH*)	Forward: 5′-AATATCATCCCTGCTTCTACTGG-3′; reverse: 5′-CATACTTGGCAGGTTTCTCCA-3′	gi:77404272	154	57	6.1[b]
Cyclooxygenase-2	Forward: 5′-ATGTATCCTCCCACTGTCAAAG-3′; reverse: 5′-TGTTCCCGCAGCCAAATG-3′	gi:1703495	146	53	10.1
Aldoketoreductase 1B5	Forward: 5′-AAGTGGTGAAGCCTGAGG-3′; reverse: 5′-CAGTGGATGAGGTAGAGGTC-3′	gi:265403	138	57	11.3
Prostaglandin 15-dehydrogenase	Forward: 5′-ACCTACCTGGGCTTGGATTAC-3′; reverse: 5′-CTGCCGAGCGTGTGAATC-3′	gi:4033852	150	64	9.3
Prostaglandin E synthase	Forward: 5′-CAACTGAGGCTGCGGAAG-3′; reverse: 5′-CCAGGAACAGGAAGGGGTAG-3′	gi:31341986	150	62	9.8
Preproendothelin-1	Forward: 5′-ATCATCTGGGTCAACACTCC-3′; reverse: 5′-TAGCACACTGGCATCTCTTC-3′	gi:31341389	129	51	16.5
Preproendothelin-3	Forward: 5′-TGTGCCAGCGAGATGGTAT-3′; reverse: 5′-GCCCTAAGGAACACAAGCTG-3′	gi:119905653	155	59	6.5
Endothelin converting enzyme-1	Forward: 5′-GCATCCAATACCAGACAAGAAC-3′; reverse: 5′-ACAGGCATAGGTGAAGAAGTC-3′	gi:31341344	127	58	16.3
Endothelin receptor-A	Forward: 5′-TGTCCTTCTGGGTGGCTCTG-3′; reverse: 5′-TTCGTGGGTTGATGTGTGGTG-3′	gi:27805816	144	54	6.9
Endothelin receptor-B	Forward: 5′-GCAGGATTTTGAAGCTCACTC-3′; reverse: 5′-TTTTGCTCACCAAATACAGAGC-3′	gi:31342360	150	57	9.7

[a] CVs were determined following linearization of C_t values and were calculated for each sample.
[b] Value represents the average of GAPDH CV for all samples.

drogenase (*GAPDH*), were designed based on each gene sequence using Primer3 software (Table 1; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Quantitative PCR was performed in duplicate for each cDNA sample on a Bio-Rad iCycler iQ Real-Time PCR Detection System using iQTM SYBR1 Green Supermix (Bio-Rad, Hercules, CA, USA) in 25-μL reaction volumes containing 300 nM of each primer and 2 μL diluted cDNA. Standard curves for each gene and the control were constructed using six 10-fold serial dilutions of cDNA from reverse transcription of primers yielding a larger product (≈350 bp) than cDNA from samples (≈150 bp). Threshold lines were adjusted to intersect amplification lines in the linear portion of the amplification curve, and cycles to threshold (C_t) were recorded. The quantity of each mRNA was determined from the appropriate standard curve and then divided by the quantity of mRNA for *GAPDH* to obtain a normalized value.

2.2.2. Supplement to Experiment 2

Luteal expression of genes from the endothelin and prostaglandin systems during the estrous cycle was compared with Day 30 of gestation, because *AKR1B5* had lower than expected expression on Day 30 in Experiment 2. The samples obtained from Experiment 2 were compared to those from luteal tissue collected on Day 4 ($n = 6$) and Day 10 ($n = 8$) of the estrous cycle from a separate group of mature, non-lactating cows. Gene expression was quantified by the same method and standard curves described above, with all samples being examined concurrently.

2.2.3. Incubation of luteal cells

Dispersed luteal cells were obtained as described by Milvae et al. [38] and used to determine effects of serum concentration of progesterone at lutectomy on *in vitro* production of progesterone and prostaglandins. For analysis of net biosynthesis of progesterone, 1×10^5 cells were incubated, in quadruplicate, in 500 μL of media with treatments for 2 h and frozen at the end of incubation. Treatments included: control, 5 ng bovine LH (USDA bLH B-5, USDA Animal Hormone Program, Beltsville, MD, USA), 1 μg arachidonic acid (AA; Sigma Chemical Co., St. Louis, MO, USA), 10^{-10} to 10^{-7} M EDN1 (Sigma Chemical Co.) and combinations of each dosage of EDN1 with LH or AA. One set of quadruplicate tubes for each CL was frozen immediately after cell dispersion for determination of pre-incubation concentrations of progesterone. Data were analyzed and reported as the difference between pre- and post-incubation values of progesterone.

For analysis of synthesis of $PGF_2\alpha$ and 6-keto-$PGF_1\alpha$ (the stable metabolite of PGI_2), 1×10^6 cells

were incubated in duplicate with treatments for 2 h and frozen at the end of incubation. Treatments included: control, 1 μg AA, 10^{-10} to 10^{-7} M EDN1, and combinations of each dosage of EDN1 with AA. One set of quadruplicate tubes for each CL was frozen immediately after cell dispersion for determination of pre-incubation concentrations of each PG. Data were analyzed and reported as the difference between pre- and post-incubation values for each PG.

2.2.4. Assays for progesterone, $PGF_2\alpha$, and 6-keto-$PGF_1\alpha$

Luteal and serum concentrations of progesterone were determined by radioimmunoassay, as described by Sheffel et al. [33]. The inter- and intra-assay coefficients of variation were 7.1 and 5.5%, respectively, and sensitivity was 12 pg/tube. Luteal tissue was homogenized in 1 mL phosphate buffered saline per milligram of tissue. The homogenate was centrifuged at 1500 × *g* for 15 min, the supernatant was diluted 1–800 in PBS, and 100 μL diluted supernatant was assayed. Frozen serum was allowed to thaw at 4 °C and 100 μL was assayed. Concentrations of progesterone, $PGF_2\alpha$, and 6-keto-$PGF_1\alpha$ in media from incubation of luteal cells were determined by radioimmunoassays [25,39,40]. The inter- and intra-assay coefficients of variation and sensitivities were: progesterone = 8.4%, 9.3%, and 25 pg/tube; $PGF_2\alpha$ = 9.8%, 10.4%, and 4 pg/tube; and 6-keto-$PGF_1\alpha$ = 11.1%, 9.7%, and 1.4 pg/tube.

2.3. Statistical analyses

All data were distributed normally in each experiment [41]. In Experiment 1, mean concentrations (ng/mL) of progesterone varied more within than between treatments with two new or two used CIDR inserts on Days 28–38. Therefore, mean concentrations of progesterone for each cow during Days 30–33 or 30–38 were included in the statistical model instead of type of CIDR. Associations of pregnancy status on either Day 38 or 53 with mean concentrations of progesterone or estradiol from Days 30 to 33 or 30 to 38, age of cow, body condition score, growth rate of the embryo (mm/d), and diameter of the largest follicle present between Days 30 and 38 were determined using logistic regression. The growth rate of each embryo, change in crown-rump length of the embryo per day, was calculated for two time periods, Days 28–38 (or the last day an embryo was viable) and Days 28–53 (or the last day an embryo was viable). Associations of pregnancy status on Day 38 with growth rate of embryos from Day 28 to 38, and of pregnancy status on Day 53 with either the growth rate from Days 28 to 53 of all embryos ($n = 40$), or only those embryos that were viable on Day 38 ($n = 33$), were evaluated. One cow lost both CIDR inserts after Day 45 and lost pregnancy; that cow was excluded from analyses after Day 38. All analyses were performed by SAS® software [42].

In Experiment 2, total luteal progesterone, luteal concentration of progesterone, and luteal concentrations of mRNA's were examined for relationship to progesterone in serum at Hour 0 by linear regression. Data for net synthesis of progesterone, $PGF_2\alpha$, and 6-keto-$PGF_1\alpha$ were examined by ANOVA, using pre-incubation concentrations as covariates, for effects of classification of the cow based upon concentration of progesterone in serum at Hour 0 above or below the overall mean (High or Low, respectively), treatments during incubation and interaction of classification and treatment. Each breed of cow was represented in both classes: High, $n = 3$ Ayrshire, 3 Holstein; Low, $n = 2$ Ayrshire, 4 Holstein. Concentrations of progesterone in serum decreased approximately 80% during the first hour, and were below 0.5 ng/mL by 6 h after removal of CL from heifers [43]. Therefore, Hour 0 values were not included in analysis of patterns of serum progesterone after lutectomy, so that measured concentrations of progesterone in response to exogenous treatment would not be biased by the initial endogenous progesterone. Patterns of progesterone in serum were compared by ANOVA, with classification of progesterone at Hour 0 (High vs. Low) in the main plot and time by classification in the sub-plot [44]. Areas under the curve for serum progesterone from 4 to 48 h after lutectomy were compared by analysis of covariance, with concentration of progesterone at Hour 0 as a covariate.

3. Results

3.1. Experiment 1

Among all cows, concentrations of progesterone increased 1.0 ± 0.2 ng/mL from Days 28 to 29 following placement of CIDR inserts, and decreased 2.3 ± 0.3 ng/mL from Days 29 to 30, after lutectomy (Fig. 1). Thereafter, mean concentrations of progesterone averaged 1.8 ± 0.1 ng/mL. Mean concentrations of estradiol between Days 30 and 38 were 2.3 ± 0.1 pg/mL (Fig. 1).

Pregnancy was lost from nine cows during progesterone replacement with CIDR inserts. Retention of pregnancy (85% to Day 38 and 77% to Day 53) was

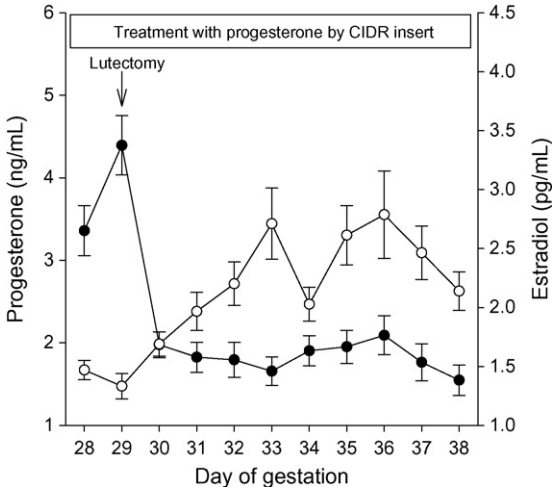

Fig. 1. Concentrations of progesterone (ng/mL, ●) or estradiol (pg/mL, ○) on Days 28–38 of gestation in suckled beef cows treated with two new or two used CIDR inserts, replaced every 5 d after lutectomy on Day 29 (mean ± S.E.M.).

lower ($P < 0.05$) for treated than for untreated control cows (97.3% to Day 53). Sixty-six percent of the pregnancy loss occurred between Days 28 and 38. Retention of pregnancy to Day 38 or 53 decreased with age of the cow ($P < 0.05$). Crown-rump length of the embryos increased from 9.3 ± 0.3 mm on Day 28 to 19.6 ± 0.4 mm on Day 38. Although retention of pregnancy to Day 38 was not associated with growth rate of the embryos from Days 28 to 38, retention of pregnancy to Day 53 was associated positively with growth rate of the embryos from Days 28 to 53 for all cows ($P = 0.02$). Retention was not associated with individual concentrations of progesterone (range 0.7–5.0 ng/mL) or estradiol (range 1.2–4.2 pg/mL) on Days 30–33, or with diameter of the largest follicle (range 8–17 mm) or body condition score (range 4–8). Average serum progesterone was greater ($P < 0.05$; 2.3 ng/mL) for untreated controls as compared to the treated group (1.7 ng/mL).

After Day 34 and before Day 38, a new CL was identified by ultrasonography in five cows, in each case on the ovary ipsilateral to the uterine horn of pregnancy. All five were older cows (three with used and two with new CIDR inserts). Concentrations of progesterone in these cows began to increase as early as Day 33, above those recorded for the remainder of the cows, to concentrations found normally in pregnant cows. The new CL and pregnancy were maintained to Day 53 in three of these cows. The lifespan of the CL from spontaneous luteinization was transient in the remaining two cows and it was no longer visible by ultrasonography on Day 40; one lost pregnancy and the other maintained pregnancy to Day 53.

3.2. Experiment 2

3.2.1. Serum progesterone concentrations post-lutectomy

Patterns of concentrations of progesterone in serum after CL removal differed ($P < 0.0001$) between cows classified as High or Low in progesterone at lutectomy. Concentrations were greater and increased more after 36 h for cows in the High than in the Low group during twice-daily injections of exogenous progesterone (150 mg SC; Fig. 2). Area under the curve for serum progesterone, not including Hour 0 values, ranged from 36 to 125 arbitrary units for individual cows and was greater ($P = 0.03$) for the High (83.6 ± 12.5) than Low (49.6 ± 6.2) group. Area under the curve increased as Hour 0 progesterone in serum increased ($b = 0.74$; $P = 0.006$). Plasma concentrations of progesterone, either before or after lutectomy, were not associated with milk production or breed of cow.

3.2.2. Luteal progesterone and gene expression

Two-dimensional diameter of the CL at ultrasonography (mean = 22.4 ± 0.5 mm), luteal wet weight (mean = 8.55 ± 0.8 g), and total luteal progesterone content (mean = 106.0 ± 3.3 μg) and concentration (mean = 12.7 ± 0.5 μg/g) did not differ between High

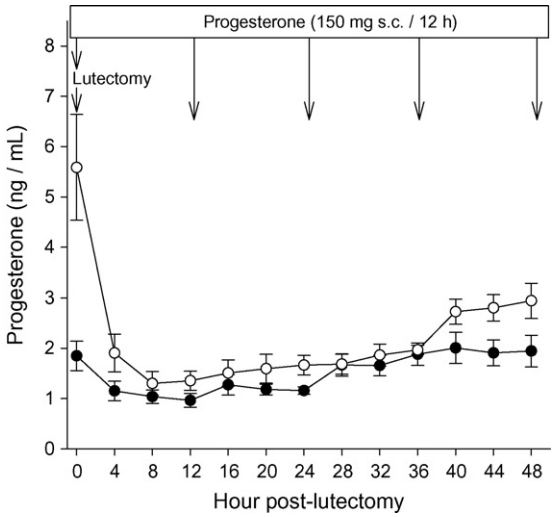

Fig. 2. Patterns of progesterone (ng/mL) in serum, after lutectomy, for pregnant lactating dairy cows with High (○) or Low (●) circulating concentrations of progesterone before lutectomy (mean ± S.E.M.) that were treated with 150 mg progesterone every 12 h beginning at lutectomy. Patterns from 4 through 48 h differed ($P < 0.0001$), as did area under the curve ($P = 0.03$).

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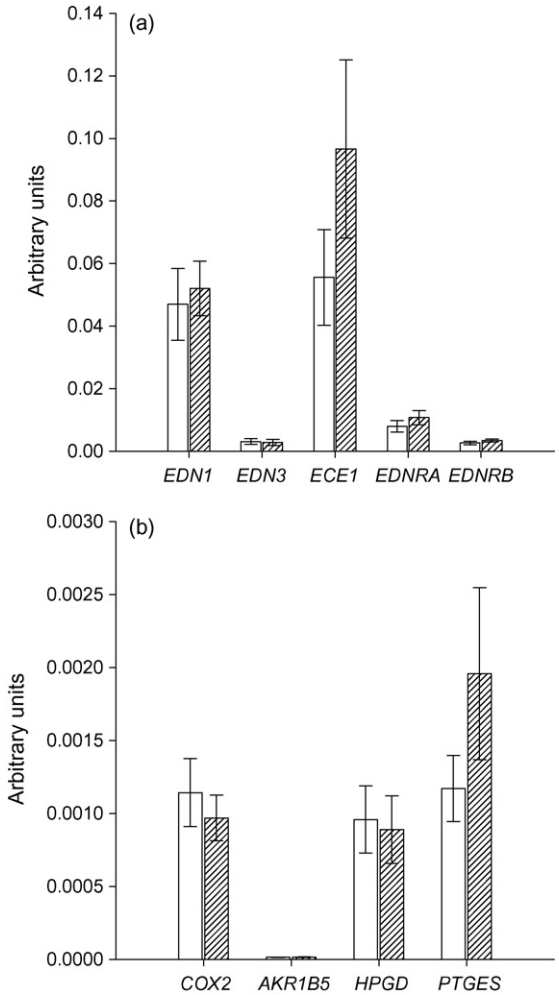

Fig. 3. Relative abundances of mRNA in CL of cows with High (open bars) or Low (hashed bars) circulating concentrations of progesterone (means ± S.E.M.). Panel a: preproendothelin-1 (*EDN1*), preproendothelin-3 (*EDN3*), endothelin converting enzyme (*ECE1*), endothelin receptor A (*EDNRA*), and endothelin receptor B (*EDNRB*). Panel b: cyclooxygenase-2 (*COX2*), aldoketoreductase 1B5 (*AKR1B5*), hydroxyprostaglandin dehydrogenase 15 (*HPGD*), and prostaglandin E synthase (*PTGES*). For cows in both groups combined, *EDN1* was expressed at a greater ($P < 0.05$) abundance than *EDN3* and *EDNRA* was more ($P < 0.05$) abundant than *EDNRB*. Expression of *AKR1B5* was lower ($P < 0.05$) relative to all other prostaglandin genes measured.

and Low cows. Neither content nor concentration of progesterone in CL was associated with Hour 0 concentrations of progesterone in serum ($b = 0.36$ and 0.11, respectively). Total luteal progesterone was not associated with luteal wet weight or size at ultrasonography ($b = 0.24$ and 0.31, respectively). Luteal contents of mRNA for enzymes from the endothelin and prostaglandin pathways were not associated with Hour 0 progesterone and did not differ between groups (Fig. 3a and b). For all cows, mRNA for *EDN3* was less abundant than *EDN1* and *EDNRA* was more abundant than *EDNRB* (Fig. 3a). Expression of *AKR1B5* was relatively low compared to other genes of the prostaglandin system (Fig. 3b).

3.2.2.1. Supplement to Experiment 2. Expressions of *EDN3*, *COX2* and *AKRB5* were similar for all three of the days examined. Abundance of *PGES* was greater ($P < 0.05$) on Day 4 than on Day 10 of the estrous cycle and Day 30 of pregnancy, which were similar. The relative concentration of *EDN1* was similar for Days 10 and 30, and both were greater ($P < 0.05$) than on Day 4. Abundances of mRNA for *EDNRA*, *EDNRB*, and *ECE1* were similar on Days 4 and 10 of the estrous cycle, but greater ($P < 0.05$) on Day 30 of pregnancy than during the cycle. Expression of *HPGD* was greater ($P < 0.05$) on Day 10 than on Days 4 and 30, which were similar.

3.2.3. Incubations of dispersed luteal cells

Hour 0 progesterone and net progesterone biosynthesis during 2-h incubation without treatment (control) did not differ for luteal cells from High and Low cows. Incubation with 5 ng bovine LH increased ($P < 0.05$) net biosynthesis of progesterone above control. Incubation with 1 μg AA alone did not alter biosynthesis of progesterone. Both basal and LH-stimulated secretion of progesterone by luteal cells were inhibited by EDN1 in a dose-dependent manner ($P < 0.0001$; Fig. 3a). A group effect was detected when EDN1 was added in the presence of AA; in that situation, reduction of progesterone production by EDN1 was greater ($P < 0.05$) for luteal cells from Low cows (Fig. 3b).

Initial, control, and AA-stimulated concentrations of $PGF_2\alpha$ and 6-keto-$PGF_1\alpha$ did not differ for luteal cells from High and Low cows. Concentrations of $PGF_2\alpha$ were increased ($P < 0.0001$) in a dose-dependent manner by the addition of EDN1 or EDN1 and AA to incubation media with no difference between groups (Fig. 4a). In contrast, concentrations of 6-keto-$PGF_1\alpha$ were decreased ($P < 0.0001$) in a dose-dependent manner by the addition of EDN1 or EDN1 and AA to incubation media, but again, there was no difference between High and Low cows (Fig. 4b).

4. Discussion

Progesterone or progestogen in sufficient concentration is necessary for maintenance of pregnancy in ovariectomized or lutectomized cows. In Experiment 1, reduction of progesterone concentration in beef cows promoted embryo loss (23% compared to 2.7% in

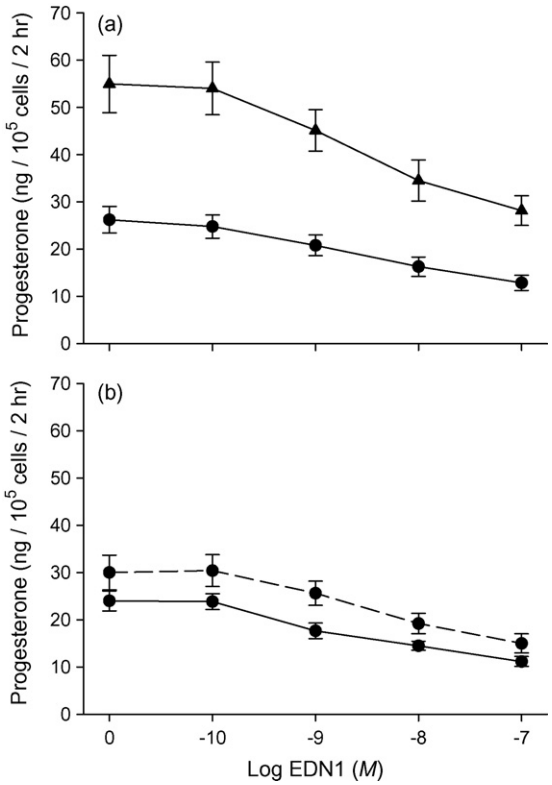

Fig. 4. Net biosynthesis of progesterone (ng/10^5 cells/2 h) by dispersed luteal cells. Panel a: because a group effect of low vs. high progesterone at lutectomy was not observed, points represent means ± S.E.M. for cows in both groups combined when cells were incubated with increasing concentrations (10^{-10} to 10^{-7} M) of endothelin-1 (EDN1) and either no additional treatment (●) or 5 ng bovine luteinizing hormone (▲). Panel b: means ± S.E.M. for luteal cells from cows with High (- - -●- - -) or Low (— ● —) progesterone incubated with increasing concentrations (10^{-10} to 10^{-7} M) of ET and 1 μg arachidonic acid (AA). Reduction of progesterone by EDN1 was greater ($P < 0.05$) for luteal cells from Low cows when incubated with AA.

control cows in the herd, comparable to reported values of 2–6%) [4]. Most losses occurred in the late embryonic period (67%) compared to the early fetal period (33%). Had concentrations of progesterone been more variable among the experimental cows, a relationship of pregnancy retention to individual concentrations of progesterone might have been detected. New and used CIDR inserts did not provide the expected variability, but rather peripheral progesterone was uniformly low in treated cows that did not form new CL. Most of the variation that occurred was due to endogenous production by CL that formed spontaneously after lutectomy in five cows. For these cows, the precipitous decline in progesterone at lutectomy must have allowed sufficient release of gonadotropins to initiate lutenization of a large follicle present at the time. However, only three of those five CL were capable of survival and support of continued pregnancy. In that respect, performance of these CL was consistent with that of CL induced before Day 36 of gestation in lutectomized cows [37]; only 50% maintained the pregnancy in that study.

Beef cows did not appear to be as sensitive to reduction in concentrations of progesterone as dairy cows [5], in which concentrations below 2.8 ng/mL led to loss of 50% of pregnancies. Nevertheless, the present results, along with the improved retention of pregnancies with supplemental progesterone during the early fetal period in dairy cows [45], confirmed that peripheral progesterone is an important indicator of embryonic/fetal survival during placentation.

Heifers and non-lactating dairy cows had equivalent or greater concentrations of peripheral progesterone despite having smaller CL than lactating dairy cows [30]. Neither luteal size nor wet weight affected peripheral concentrations of progesterone in the present study. Although ovarian effluent concentrations of progesterone were related to luteal content in cycling ewes [46], peripheral concentration was not related to luteal content in cycling dairy cows [47]. Similarly, luteal content and concentration were not associated with peripheral concentrations in these cows. Therefore, ovarian blood flow [48] and catabolism of progesterone, either jointly or independently, must be more important regulators of peripheral concentrations than secretion. When given exogenous progesterone after lutectomy, cows in the High group maintained greater concentrations of progesterone than those in the Low group. Thus, systemic catabolic factors affected peripheral concentrations of progesterone more than either ovarian blood flow [48] or luteal production.

Expression of mRNAs for genes involved in the endothelin and prostaglandin systems did not differ for luteal tissue from High and Low cows. The mRNA for *EDN3* was less abundant than *EDN1* and *EDNRA* was more abundant than *EDNRB*. These data corresponded well with previous reports of relative expression of genes from the endothelin family in cow CL [16,17].

Interestingly, the expression of *AKR1B5*, the prostaglandin F synthase (*PGFS*) considered important for synthesis of $PGF_2\alpha$ in the cow CL during luteal regression [29], was relatively low compared to other genes of the prostaglandin system measured in this experiment. If that were the case, it could be an important indicator that capacity for luteal synthesis of prostaglandin $F_2\alpha$ was reduced during pregnancy. However, that concept did not fit with the earlier data of Schallenberger et al. [49], nor that of Bridges et al.

[37], that an increase in prostaglandin $F_2\alpha$ was to be expected during Days 30–35 and might be important to the process of attachment. Therefore, the supplement to Experiment 2 was added to determine whether this apparently low expression was an effect of pregnancy. Expression of *AKR1B5* did not differ among CL from early- and mid-cycle or Day 30 of gestation. Other reports have recorded changes in its expression under differing physiological conditions. Costine et al. [50] reported that the expression of *AKR1B5* was similar in the CL of pregnant and non-pregnant ewes before $PGF_2\alpha$ injection on Day 12 post-estrus, but that its expression decreased in non-pregnant, but not in pregnant ewes 4 h after injection of $PGF_2\alpha$. Abundance of *AKR1B5* mRNA in CL of cows was greater on Day 16 than on Day 5 and greater in Day-5 short-lived CL than in Day-5 normal CL [51]. Arosh et al. [29] found a shift in expression of *AKR1B5* relative to expression of *PGES* that favored *PGES* in cow CL during Days 10–13 of the estrous cycle.

Increased expression of *EDN1*, *EDNRA*, *EDNRB*, and *ECE1*, on Day 10 of the cycle and Day 30 of pregnancy, compared to Day 4 of the cycle, might indicate maturation and increased vascular tissue of the growing or maintained CL. Greater expression of *PGES* on Day 4 compared to Days 10 and 30 supports an early luteotropic role for PGE_2 [25,40,51]. The increased expression of *HPGD* on Day 10 fits the scenario of increased degradation of $PGF_2\alpha$ before the luteolytic cascade is initiated [52].

Net biosynthesis of progesterone *in vitro* was similar between High and Low cows whether un-stimulated or driven with LH. Likewise, factors affecting luteal synthesis of progesterone did not differ. However, in the presence of arachidonic acid, luteal cells from Low cows were more susceptible to EDN1 than cells from High cows. Addition of AA to incubated luteal cells in culture increased secretion of anti-steroidogenic $PGF_2\alpha$, which might have shifted the enzymatic conversion away from the luteotropic prostaglandin, PGI_2, as indicated by decreased accumulation of its metabolite 6-keto-$PGF_1\alpha$ (Fig. 5). However, cells from High and Low cows did not differ in stimulation of $PGF_2\alpha$ or inhibition of PGI_2 by EDN1.

Corpora lutea from pregnant ewes on Day 14 had a greater capacity to catabolize $PGF_2\alpha$ into 13,14 dihydro-15-keto $PGF_2\alpha$ than CL from non-pregnant ewes in spite of the fact that mRNA expression for *PGDH* did not differ [50]. However, the hypothesis that increased PGDH activity was responsible for the difference in secretion of progesterone by luteal tissue from cows with High or Low peripheral progesterone in

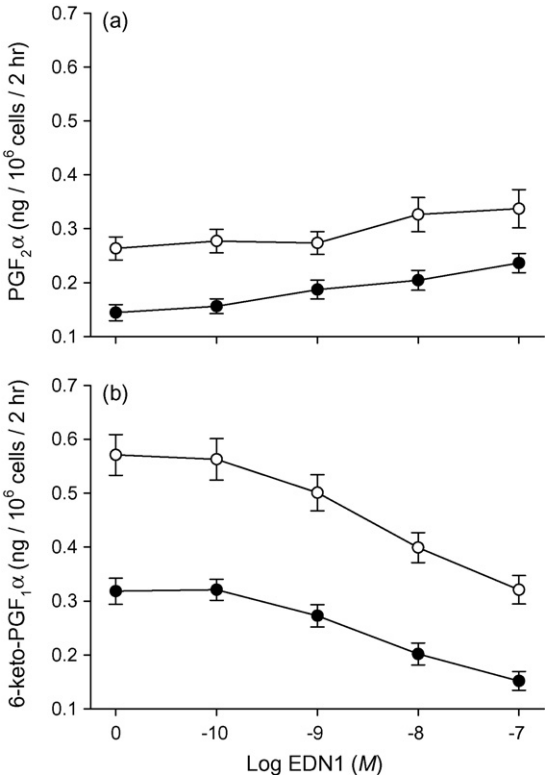

Fig. 5. Net production (ng/10^6 cells/2 h) of prostaglandin $F_2\alpha$ (a, $PGF_2\alpha$) and 6-keto-$PGF_1\alpha$ (b) by dispersed luteal cells incubated with increasing concentrations of endothelin-1 (EDN1, 10^{-10} to 10^{-7} M) and either without additional treatment (●) or with 1 μg arachidonic acid (AA, ○). Because a group effect (Low vs. High progesterone at lutectomy) was not detected, points represent means ± S.E.M. for cows in both groups combined.

response to EDN1 and AA in this study was not supported; groups did not differ in either luteal mRNA for *PGDH* or luteal secretion of $PGF_2\alpha$. Therefore, we inferred that luteal secretion of progesterone, and the systems known to alter it, function similarly for cows with High or Low peripheral serum concentrations of progesterone during gestation. Changes in factors that modulate luteal function throughout the estrous cycle did not appear to explain lower serum concentrations of progesterone in some pregnant cows at approximately Day 30 of gestation.

The present data supported the hypothesis that low circulating concentrations of progesterone, known to affect fertility among lactating dairy cows, are an indirect consequence of altered nutritional demands. It is reasonable that the factors controlling rate of steroid clearance for lactating dairy cows remained similar during the estrous cycle and early gestation. Dry matter and energy intake were not controlled or measured in Experiment 2. That serum concentration of progester-

one before and after lutectomy was not correlated with milk production in this study might reflect the degree of variation and the relatively small number of cows studied. However, that injected progesterone supported greater circulating concentrations of progesterone in cows with greater serum progesterone prior to lutectomy compared to those with lower progesterone, provided strong evidence that metabolic clearance is responsible for lower peripheral concentrations of progesterone.

In conclusion, low concentrations of serum progesterone, in lactating dairy cows that are prone to pregnancy failure during placentation, are likely the result of increased clearance rather than decreased luteal production. More intensive research will be required to fully characterize whether increased clearance is the result of increased hepatic blood flow, increased hepatic enzymatic function [53], or both. Similarly, more work is needed to understand how lower concentrations of progesterone allow loss of pregnancy in only some cows. Poor reproductive efficiency makes this type of research cost- and time-prohibitive in lactating dairy cows. Beef cows are less expensive and experimental reduction of progesterone increased their frequency of pregnancy failure. Several factors that might confound results in dairy cows might be avoided in beef cows. For instance, large groups of beef cows commonly are inseminated at similar stages postpartum on a single day, and with greater conception rates than in dairy cows. Therefore, controlling the serum concentrations of progesterone in suckling beef cows, before and during placentation, might be a more efficient method for determining the mechanism by which low progesterone in lactating dairy cows, as a result of increased clearance, leads to greater pregnancy failure.

Acknowledgements

Appreciation is expressed to Pfizer Animal Health, New York, NY, USA for gifts of Eazi-Breed CIDR® inserts and Lutalyse®. We especially appreciate critical reading of the manuscript and important suggestions by Dr. Robert A. Dailey.

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Please cite this article in press as: Rhinehart JD, et al. Low peripheral progesterone and late embryonic/early fetal loss in suckled beef and lactating dairy cows. Theriogenology (2008), doi:10.1016/j.theriogenology.2008.07.031