Effect of 2 corpora lutea on blood perfusion, peripheral progesterone, and hepatic steroid-inactivating enzymes in dairy cattle

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ABSTRACT

The luteal structure that develops postovulation is critical to the facilitation and maintenance of pregnancy in dairy cattle. The objectives of this experiment were to determine if the induction of an accessory corpus luteum (CL), via human chorionic gonadotropin, altered blood perfusion of CL, peripheral concentrations of progesterone, or hepatic steroid-inactivating enzymes. Twenty-eight late-lactation Holstein cows were synchronized using the Ovsynch protocol and randomly assigned to 1 of 2 treatment groups. Cows received either an injection of human chorionic gonadotropin (1,000 IU, i.m.) to induce an accessory CL (cows had exactly 2 CL in 1 ovary) or no treatment (cows had exactly 1 CL). Corpora lutea were examined daily from d 10 to 18 (d 0 was induced ovulation) via Doppler ultrasonography and a blood sample was collected. Volume of the CL was recorded, as well as images and videos of each CL, which were analyzed for blood perfusion. On d 13, a liver biopsy was performed to analyze hepatic steroid-inactivating enzymes. Cows with 1 or 2 CL had similar peripheral concentrations of progesterone. Cows with 2 CL had similar luteal volumes to cows with 1 CL but cows with 2 CL had greater total luteal blood perfusion. Hepatic enzyme [cytochrome P450 (CYP) 1A, 3A, and 2C, aldo-keto reductase 1C, and uridine diphosphate glucuronosyltransferase] activities did not differ between cows with 1 and 2 CL. Overall, the observed increase in total luteal blood perfusion in cows with 2 CL did not correspond to differences in peripheral concentrations of progesterone or clearance of progesterone measured by the hepatic enzyme activity. This could indicate that induction of an accessory CL would not affect concentrations of progesterone necessary to maintain pregnancy.

Key words: blood perfusion, corpus luteum, progesterone

INTRODUCTION

Steady declines in reproductive efficiency in dairy cattle have been documented over the past 60 yr, including decreased pregnancy rates and increased embryonic mortality. Decreased concentrations of progesterone due to increased catabolism, decreased secretion, or both, could be responsible for some of this reproductive inefficiency (Inskeep and Dailey, 2005). Rhinehart et al. (2009) showed a larger role for progesterone catabolism versus luteal secretion in controlling peripheral concentrations of progesterone during pregnancy in dairy cattle.

Progesterone is catabolized in the liver by enzymes of the cytochrome P450 (CYP) family, aldo-keto reductase (AKR) family, and uridine diphosphate glucuronosyltransferase (UGT). An in vitro study utilizing primary bovine hepatic cell cultures determined that cytochrome P450 2C was the largest contributor to progesterone inactivation followed by AKR1C and CYP3A, respectively (Lemley and Wilson, 2010). Regulation of these enzymes in vivo could be dependent on the rate of substrate delivery via hepatic blood flow. Sangsritavong et al. (2002) found that greater feed intake increased hepatic blood flow and the metabolic clearance rate of progesterone.

Double ovulation is common in dairy cattle and can lead to the undesirable occurrence of twinning. Silva Del Rio et al. (2006) found that 93% of twins in dairy cattle are nonidentical, meaning that the majority are the result of a double ovulation. Cows carrying a twin pregnancy are 5.4 times more likely to experience pregnancy loss than those carrying singleton pregnancies (López-Gatius et al., 2004). The physiological mechanism for this difference in pregnancy loss still eludes researchers.

Technological advances in ultrasonography have allowed for the monitoring and quantification of blood perfusion of the corpus luteum (CL). The CL is highly vascularized and, with the use of power Doppler ultrasonography, blood perfusion can be recorded. Herzog et al. (2010) assessed the reliability of luteal blood perfusion, via power Doppler ultrasonography, in conjunction with luteal size and circulating concentra-

Received July 8, 2014.

Accepted September 15, 2014.

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tions of progesterone in cattle. They found a positive correlation between luteal blood perfusion and plasma concentrations of progesterone.

Thus, the objective of this study was to determine if the induction of an accessory CL, via human chorionic gonadotropin (**hCG**), alters blood perfusion of CL, peripheral concentrations of progesterone, or hepatic steroid-inactivating enzymes.

MATERIALS AND METHODS

All procedures in this study were approved by the Institutional Animal Care and Use Committee of Mississippi State University.

Animals and Experiment

A total of 28 late-lactation Holstein cows were housed in a freestall barn at the Bearden Dairy Research Center (Starkville, MS). Cows were allowed ad libitum access to a TMR formulated to meet or exceed dietary requirements of lactating cows (NRC, 2001). Average DIM was 289 ± 60 d (range of 163 to 399 d) and average milk production was 32.4 ± 9.7 kg/d. From mid-July to early November, cows had their estrous cycles synchronized using the Ovsynch protocol (Pursley et al., 1995), with d 0 being the day of the second injection of GnRH. Seven days after the completion of Ovsynch (d 7), ovaries were examined using transrectal ultrasonography (10.0- to 5.0-MHz linear-array transducer, MicroMaxx, SonoSite Inc., Bothell, WA). Cows that responded to Ovsynch and had a single CL remained in the study and either received an injection of hCG (1,000 IU i.m.; Chorulon, Intervet Inc., Millsboro, DE) to induce an accessory CL, or received no additional treatment. Cows that did not respond to Ovsynch or that had multiple CL at this time were excluded from the current repetition. Cows that did not respond or were excluded from the previous repetition were re-enrolled using the same process as previously stated.

On d 10, cows that had received hCG were required to have 2 CL and those that failed to produce 1 accessory CL in response to hCG were excluded from the study. Cows in the control group were also examined to ensure that the single CL had not regressed. Cows that failed to respond to the initial synchronization or failed to produce 1 accessory CL were resubmitted to Ovsynch and subsequent treatments and evaluations, with a minimum of a 15-d period between repetitions.

From d 10 to 18 or until CL regression, ovaries were mapped using transrectal ultrasonography (10.0- to 5.0-MHz linear-array transducer, MicroMaxx, SonoSite Inc.). The diameter of each CL was measured and the

volume calculated using a previously published method (Vasconcelos et al., 2001). Blood perfusion of the CL was evaluated using the power flow option of the ultrasound machine, and 2 still images and 1 video were recorded for each CL for later analysis. Blood samples were collected daily from d 10 to 18 by venipuncture of the tail in a spray-coated K₂ EDTA tube (Becton, Dickinson and Co., Franklin Lakes, NJ) and immediately placed on ice. Within 2 h of collection, tubes were centrifuged at $2,000 \times g$ at 4°C for 20 min, and plasma was frozen at -20°C until later analysis.

On d 13, a liver biopsy was performed following the published methods of Lemley et al. (2010). Briefly, hair was removed on the right side of the cow at the 10th intercostal space with a clipper, and an ultrasound scan of the liver was performed to locate and avoid large hepatic vessels during the biopsy procedure. The external area of the 10th intercostal space was scrubbed 3 times with betadine (Purdue Products L.P., Stamford, CT), and a local anesthetic (approximately 10 mL of 2% lidocaine hydrochloride; MWI, Boise, ID) was administered. After a short interval, the skin was punctured with a scalpel and the liver sample was collected using a biopsy needle machined at Mississippi State University's Department of Agricultural and Biological Engineering (Mississippi State) following the specifications of Swanson et al. (2000). After the liver sample was collected, it was placed in a cryogenic vial, snap frozen by submersion in liquid nitrogen, and stored at -80°C until analysis of hepatic enzyme activity. The incision site was closed with a small skin staple, which was sprayed with Blu-kote antiseptic (H. W. Naylor Co. Inc., Morris, NY) and removed 5 d later after the incision healed. Cows were observed and body temperatures were taken to monitor for complications. Body weights were recorded before milking on d 12 and after milking on d 13 and averaged. On d 18 after completion of daily measurements and blood sampling, all cows were administered an injection of PGF_{2α} (5 mL i.m.; Lutalyse, Zoetis Inc., Kalamazoo, MI) to regress CL present.

Liver Homogenization

Approximately 100 mg of liver tissue was placed into 1 mL of potassium phosphate (KPO₄) buffer (400 mM, pH = 7.4). Samples were then placed into a glass Dounce homogenizer with an additional 1 mL of KPO₄ buffer and homogenized. Liver homogenate samples were placed into microcentrifuge tubes and then centrifuged at $10,000 \times g$ for 10 min at 4°C. The supernatant was stored at -80°C until enzyme assays were conducted. The protein concentration of the supernatant was determined by a Coomassie Plus (Bradford) protein

assay following the manufacturer's protocol (Thermo Scientific, Rockford, IL).

CYP Enzymes

Assay kits for CYP1A, CYP3A, and CYP2C and NADPH regeneration solution were purchased from Promega Corporation (Madison, WI) and assays conducted according to Hart et al. (2014). Briefly, reconstitution buffer was added to luciferin detection reagent. Luciferin CEE (CYP1A), luciferin IPA (CYP3A), and luciferin H (CYP2C) were diluted in KPO_4 buffer. Liver homogenates (30 μg of protein per well) and enzyme-specific luciferin substrate were added to 96-well plates in duplicate. Plates were then preincubated for 10 min (CYP1A and CYP3A) or 30 min (CYP2C) at 37°C. Following the incubation, NADPH regeneration solution was added to each well and plates were incubated for 30 min (CYP1A and CYP2C) at 37°C or 10 min (CYP3A) at room temperature. After the incubation, luciferin detection reagent was added to each well and plates were protected from light and incubated for an additional 20 min at room temperature. Plates were then placed into a Promega Multi-Plus plate reader and luminescence was measured.

UGT

The UGT assay kit was purchased from Promega Corporation and the assay performed according to Hart et al. (2014). Briefly, uridine diphosphoglucuronic acid (UDPGA) was added to half the plates to act as reaction wells, and distilled water was added to the other half as control wells. The UGT reaction mixture containing UGT multienzyme substrate was then combined with liver homogenates (28 μg of liver protein per well), and the plates were preincubated for 10 min at 37°C. After incubation, detection reagent was added to each of the wells followed by an incubation period of 20 min at room temperature while protected from light. The plates were then analyzed using a Promega Multi-Plus plate reader with luminescence detection mode.

AKR1C

The activity of AKR1C was determined following the published methods of Lemley and Wilson (2010). Briefly, AKR1C was determined using the specific substrate 1-acenaphthenol (Pfaltz & Bauer, Waterbury, CT). Enzymatic reactions contained 150 μg of cytoplasmic protein, 250 μM 1-acenaphthenol, and 500 μM NADP. The 1-acenaphthenol-dependent reduction of NADP was standardized using the amount of cytoplasmic protein. The reduction of NADP was determined

by measuring the amount of light absorbed at 340 nm for 10 min using a Spectra Max Plus (Sunnyvale, CA) plate reader. The extinction coefficient for NADPH (6,220 $\text{L/mol}\cdot\text{cm}$) was used to calculate the rate of reduced NADP in picomoles per minute per milligram of protein.

RIA

Concentrations of progesterone were determined via RIA using a Coat-A-Count (Siemens Healthcare Diagnostics Inc., Los Angeles, CA) kit and following the manufacturer's protocol. Briefly, standards or 100 μL of plasma were added, in duplicate, to the coated tubes. Next, 1 mL of I-125 progesterone tracer was added to each tube. Following the 3-h incubation, tubes were aspirated and allowed to drain thoroughly; activity was assessed with a gamma counter (Packard Instrument Company, Meriden, CT). The intraassay coefficient of variation was 3.2%.

Image and Video Analysis

Images and videos were uploaded from the ultrasound machine and saved to an external location. Images were then analyzed using ImageJ software (version 1.47, US National Institutes of Health, Bethesda, MD) for integrated density to quantify total pixels of blood perfusion. Images and videos were visually scored by 2 independent and trained technicians using a scale from 0 to 9 (0 = 0% perfusion, 9 = 100% perfusion). Images and videos were randomized and presented to technicians with no additional information of cow identification, day, or treatment.

Statistical Analyses

Treatments were randomly assigned to cows, which were the experimental unit, with the exception of cows that were re-enrolled and responded successfully ($n = 5$); in those cases, the opposite treatment was assigned. Eleven cows in the control group and 8 cows in the hCG group successfully completed the study. The concentration of progesterone, total luteal volume, and blood perfusion of the CL were analyzed using repeated measurements in the MIXED procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC) with autoregressive order-1 as the covariate structure. The model contained cow, which was considered a random variable, and milk production, DIM, and BW, which were considered covariates. The treatment effect for hepatic enzymes was analyzed using the MIXED procedure of SAS. Means were separated using the PDIF option of the LSMEANS statement. Pearson correlation coef-

ficients were determined using the CORR procedure of SAS. Least squares means and pooled standard errors are reported. Statistical significance was declared at $P < 0.05$ and a tendency was declared when $P > 0.05$ but ≤ 0.10 .

RESULTS

The response rate for cows producing a single CL on d 7 was 63.3% (38 of 60 cows). Cows administered hCG produced an accessory CL 36% (9 of 25 cows) of the time. Peripheral concentrations of progesterone were not different ($P = 0.62$) between cows with 1 CL (7.19 ± 0.71 ng/mL) and cows with 2 CL (7.73 ± 0.84 ng/mL). We detected an interaction ($P = 0.02$) between treatment and day for total luteal volume (Figure 1). On d 10 to 18, total luteal volumes did not differ between treatment groups within a given day.

Total integrated densities were greater ($P = 0.001$) in cows with 2 CL ($7,781.44 \pm 758.06$ pixels) than cows with 1 CL ($4,399.69 \pm 638.95$ pixels). Similarly, blood perfusion scores were greater ($P < 0.001$) in cows with 2 CL (6.96 ± 0.32) than in cows with 1 CL (3.82 ± 0.27) for visually scored images. We detected a treatment by day interaction ($P = 0.02$; Figure 2) for videos visually scored for blood perfusion. Blood perfusion measured from videos was different ($P < 0.001$) between treatment groups for d 10 through 17 but tended to be different ($P = 0.10$) on d 18.

Blood perfusion was adjusted using the ratio of perfusion per volume. The interaction for integrated density per cubic centimeter is shown in Figure 3. Perfusions for integrated density per cubic centimeter were different between treatment groups on d 12, 15, 16, and 18

Figure 1. Total luteal volume between cows receiving control (1 corpus luteum, $n = 11$) or human chorionic gonadotropin (hCG; 2 corpora lutea, $n = 8$) treatments were similar. There was an interaction ($P = 0.02$) between treatment and day for total luteal volume. The x-axis is experiment day, with d 0 being the time of the second injection of GnRH (induced ovulation) during ovulation synchronization.

Figure 2. Visual characterization of total perfusion from images on d 10 to 18, between cows receiving control (1 corpus luteum, $n = 11$) or human chorionic gonadotropin (hCG; 2 corpora lutea, $n = 8$) treatments. A treatment by day interaction of $P \leq 0.02$ was observed. *Indicates days when treatments were different ($P \leq 0.05$). The x-axis is experiment day, with d 0 being the time of the second injection of GnRH (induced ovulation) during ovulation synchronization.

($P < 0.02$). Perfusion scores per cubic centimeter for images ($P = 0.009$) and videos ($P < 0.001$) were different between treatment groups (Figure 4). Perfusion scores per cubic centimeter for videos were also different by day ($P = 0.003$). In all instances of differences, cows with 2 CL had increased perfusion compared with cows with 1 CL.

The activity of hepatic steroid-inactivating enzymes CYP1A, CYP3A, CYP2C, AKR1C, and UGT (Table 1) did not differ ($P > 0.18$) between cows with 1 CL and those with 2 CL based on milligrams of protein and per kilogram of BW on d 13.

Figure 3. Volume adjusted integrated density perfusion from images on d 10 to 18, between cows receiving control (1 corpus luteum, $n = 11$) or human chorionic gonadotropin (hCG; 2 corpora lutea, $n = 8$) treatments. *Indicates days when treatments were different ($P \leq 0.05$). The x-axis is experiment day, with d 0 being the time of the second injection of GnRH (induced ovulation) during ovulation synchronization.

Table 1. Hepatic cytochrome P450 (CYP), aldo-keto reductase (AKR), and uridine diphosphate glucuronosyltransferase (UGT) activities in cows receiving no treatment (control) or receiving an injection of human chorionic gonadotropin (hCG) to induce an accessory corpus luteum

Enzyme ¹	Control (n = 11)	hCG (n = 8)	SE	P-value
CYP1A				
RLU/(min·mg of protein) × 10 ⁴	44.26	35.09	4.74	0.18
RLU/(min·kg of BW)	20.46	15.95	2.41	0.19
CYP2C				
RLU/(min·mg of protein) × 10 ³	4.58	4.60	0.39	0.98
RLU/(min·kg of BW)	0.21	0.21	0.02	0.90
CYP3A				
RLU/(min·mg of protein) × 10 ⁵	15.76	14.80	1.55	0.66
RLU/(min·kg of BW)	73.23	67.48	8.42	0.63
AKR1C				
pmol/(min·mg of protein)	48.39	43.39	2.77	0.21
pmol/(min·kg of BW) × 10 ³	24.49	20.69	2.00	0.19
UGT				
RLU/(min·mg of protein) × 10 ⁴	98.45	95.33	11.30	0.84
RLU/(min·kg of BW) × 10 ⁸	6.49	6.12	0.81	0.74

¹CYP1A, CYP2C, CYP3A, and UGT activity is expressed in relative light units (RLU).

Progesterone tended to be positively correlated with total luteal volume ($P = 0.10$; Table 2) but tended to be negatively correlated with milk production ($P = 0.06$; Table 2). Image scores, video scores, and integrated density were all positively correlated with each other ($P < 0.004$; Table 2) but perfusion scores were not correlated with concentrations of progesterone. Integrated density was positively correlated with total luteal volume ($P = 0.04$; Table 2) but image and video perfusion scores were not correlated to total luteal volume. Cytochrome P450 3A was negatively correlated with total luteal volume ($P < 0.05$), but no other hepatic enzymes investigated were correlated with luteal volume, progesterone, or CL blood perfusion.

DISCUSSION

Expected synchronization rates in dairy cattle using the Ovsynch protocol are approximately 87% (Vasconcelos et al., 1999). In the current study, response rates using Ovsynch were well below expected outcomes (63.3 vs. 87%). Success rates in previous reports using hCG to cause a spontaneous ovulation and induce an acces-

sory CL were approximately 70% (Wallace et al., 2011; Stevenson and Pulley, 2012). The observed response in the current study to hCG to induce an accessory CL (36%) was also less than expected. These poor responses could be due to the high ambient temperatures and relative humidity that are experienced in the southeastern United States during the summer months.

Twinning rates are positively correlated with double ovulation rates. The occurrence of twinning is between 2.4 and 5.8% in dairy cattle (Wiltbank et al., 2000), with 93% of twins being nonidentical (Silva Del Rio et al., 2006). The incidence of double ovulation, however, is more common than twinning and is reported to be between 5 and 28% (Fricke and Wiltbank, 1999; López-Gatius et al., 2005; Mann et al., 2007). Beerepoot et al. (1992) estimated that twin births cost producers, on average, \$171 more per incidence compared with singleton births. Understanding the mechanisms and physiological changes involved with double ovulation could help decrease the incidence of twinning in dairy cattle, with a positive economic impact for producers.

Similar to results in the current study, Mann et al. (2007) determined that dairy cows with a double ovula-

Table 2. Pearson correlations (r) between measurements on d 13

Item	Progesterone	Integrated density	Image score
Progesterone		0.19	0.25
Total luteal volume	0.39†	0.47*	0.28
Milk production	-0.44†	0.10	-0.14
Integrated density	0.19		0.69*
Image score	0.25	0.69*	
Video score	0.05	0.64*	0.88*

* $P < 0.05$; † $P < 0.10$.

Figure 4. Mean perfusion scores adjusted for corpus luteum volume for images (white bar) and videos (black bar) between cows receiving control (1 corpus luteum, $n = 11$) or human chorionic gonadotropin (hCG; 2 corpora lutea, $n = 8$) treatments. Perfusion scores per cubic centimeter for images ($P = 0.009$) and videos ($P < 0.001$) were different between treatment groups.

tion had similar total luteal weights and concentrations of progesterone. In contrast, Lopez et al. (2005) found that dairy cows with a double ovulation had increased luteal volume but decreased concentrations of progesterone compared with cows with a single ovulation. In beef cattle genetically selected for twinning, Echternkamp et al. (2009) found that cows with a double ovulation had greater total luteal volume and concentrations of progesterone compared with cows with a single ovulation. Interestingly, Sanjabi ewes with a double ovulation had decreased luteal volume but increased concentrations of progesterone compared with ewes with a single ovulation (Shabankareh et al., 2009). Although the size of the hCG-induced ovulatory follicle was not measured, it is expected that it would be smaller than that of a naturally occurring ovulation. A reduction in the size of the ovulatory follicle decreases the size of the subsequent CL (Vasconcelos et al., 2001) and might explain the lack of difference in total luteal volume between cows with 1 CL and cows with 2 CL in the current study.

Luteal volume and luteal blood perfusion can be used as indicators of luteal function and concentrations of peripheral progesterone (Herzog et al., 2010). Herzog et al. (2010) found a positive correlation between luteal volume and concentrations of progesterone ($r = 0.69$) as well as a positive correlation between luteal blood flow and concentrations of progesterone ($r = 0.71$). In the current study, total luteal volume only tended to be positively correlated with concentrations of progesterone. Furthermore, CL blood perfusion was not correlated with concentrations of progesterone. Despite the increase in perfusion observed in cows with 2 CL in this study, the total luteal volume was similar between the groups.

The system of visually scoring CL blood perfusion is a fairly novel idea, whereas the use of imaging software to determine pixel quantity has been documented previously (Ginther and Utt, 2004; Herzog et al., 2010). In the current study, we found no difference between perfusion scores obtained from images versus videos; therefore, either images or videos can be effective sources for visually scoring CL blood perfusion. The positive correlation between the scores obtained from images and videos with integrated density supports that visual scoring, just like pixel quantification, is an effective way of determining blood perfusion.

Inadequate peripheral concentrations of progesterone are believed to be one underlying cause of early embryonic mortality in high-producing dairy cows (Wiltbank et al., 2006). In the current study, hepatic enzymes were not altered by an induced accessory CL, but it is important to note that we observed no difference in concentrations of progesterone between treatments. In previous studies, an increase in hepatic blood flow increased the metabolic clearance rate of steroid hormones, such as progesterone, thus decreasing the peripheral concentrations (Sangsritavong et al., 2002). Rhinehart et al. (2009) showed a greater role of inactivation than secretion of progesterone in controlling peripheral concentrations. Based on results from the current experiment, we cannot conclude that induction of an accessory CL changes peripheral concentrations of progesterone and thus may not affect embryonic mortality via this mechanism.

CONCLUSIONS

Overall, the increased blood perfusion of CL in cows with 2 CL did not correspond to increased peripheral concentrations of progesterone or increased clearance as measured by hepatic enzyme activity, perhaps indicating that a double ovulation does not affect progesterone necessary to maintain pregnancy. More research is necessary to determine the underlying cause of decreased fertility and early embryonic mortality in dairy cattle with singleton or twin pregnancies.

ACKNOWLEDGMENTS

The authors thank the staff at the Joe Bearden Dairy Research Center (Mississippi State) and students A. M. Muth-Spurlock, K. E. Brockus, and C. A. Freeman for their assistance in data collection. This project was supported by the MAFES Special Research Initiative at Mississippi State University. Article is approved for publication as Journal Article No 12544 of the Mississippi Agricultural and Forestry Experiment Station, Mississippi State University.

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