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Effect of Double Ovulation on Peripheral Concentrations of Progesterone, Luteal Blood Perfusion and Hepatic Steroid Inactivating Enzymes

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By

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Progesterone is essential for the maintenance of pregnancy in cattle. Recent trends in decreased reproductive efficiency in dairy cattle have led researchers to believe that increased catabolism and decreased peripheral concentrations of progesterone are at fault. The objective of this study was to determine if the induction of an accessory corpus luteum (CL), via human chorionic gonadotropin (hCG), alters blood perfusion of CL, peripheral concentrations of progesterone, or hepatic steroid inactivating enzymes. We hypothesized that the induction of an accessory CL would decrease blood perfusion of the CL, decrease peripheral concentrations of progesterone, and increase clearance of progesterone in the liver. Total blood perfusion of the CL was increased in cows with 2 CL compared to cows with 1 CL, but concentrations of progesterone and hepatic enzymes did not differ. Overall, the increased blood perfusion in cows with 2 CL did not alter concentrations of progesterone or progesterone clearance.

Key Words: corpus luteum, dairy cattle, progesterone

DEDICATION

I would like to dedicate this thesis to my parents, Dale and Rebecca (Becky) Voelz. Thank you for all the love, encouragement, and support throughout my masters program at Mississippi State University. I love you more than anything and could not have done this without you. Thank you!

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CHAPTER I

REVIEW OF LITERATURE

Progesterone

In 1933, progesterone was first isolated and characterized in crystalline form by Wintersteiner and Allen (1934). Progesterone is a steroid hormone essential for the maintenance of pregnancy (McDonald et al., 1952) and regulation of the estrous cycle length in cattle. Progesterone is primarily secreted by the corpus luteum (CL) in the ovary and the placenta during pregnancy, but also the adrenal gland. Progesterone biosynthesis occurs by utilizing three sources of cholesterol including high-density lipoprotein, low-density lipoprotein, or cholesterol esters. Cholesterol is transported from the outer to the inner mitochondria by steroidogenic acute regulatory protein (StAR; Stocco et al., 2000). Once inside the mitochondria, cholesterol is converted into pregnenolone by cytochrome P-450 side-chain cleavage enzyme (P-450scc). Pregnenolone is then transported outside the mitochondria, where it is converted by 3β-hydroxysteroid dehydrogenase (3β-HSD), located in the smooth endoplasmic reticulum, into progesterone (Niswender et al., 2000).

Progesterone has various target tissues including the hypothalamic-pituitary axis, reproductive tract, and mammary gland. In the uterus, progesterone acts on the endometrium causing cell differentiation, stimulates glandular secretion, and changes proteins secreted by the endometrium cells (Maslar et al., 1986). These changes, ultimately, prepare the uterine environment for attachment and early embryonic

development. Less than necessary concentrations of progesterone are responsible for increased embryonic mortality and decreased maintenance of pregnancy in cattle (Butler, 2000; Wiltbank et al., 2006).

Progesterone Inactivation

The liver contains several enzymes that play an important role in the catabolism of progesterone. The first phase of progesterone inactivation involves the addition of a hydroxyl group via the cytochrome P450 and aldo-keto reductase families. Cytochrome P450 2C (CYP2C; Murray, 1991), cytochrome P450 3A (CYP3A; Murray, 1992) and aldo-keto reductase 1C (AKR1C; Penning et al., 2000) are the major enzymes involved in this process. The largest enzyme contributors to progesterone inactivation in vitro are CYP2C followed by AKR1C and CYP3A, respectively (Lemley and Wilson, 2010). The second phase of progesterone inactivation involves uridine diphosphate-glucuronsyltransferase (UGT) enzymes, which conjugate the hydroxysteroid metabolites with glucuronic acid, to create more hydrophilic metabolites for excretion. These metabolites are then excreted through feces, urine, and milk. It is still unknown if the metabolites produced by progesterone catabolism are still able to bind to receptors and have the functional properties of progesterone or if they are truly inactive.

Circulating concentrations of progesterone are a result of the rate of progesterone secretion from the CL and inactivation by liver enzymes (Inskeep and Dailey, 2005). In a recent study by Rhinehart et al. (2009), pregnant (d 28 to 34) lactating dairy cows were classified as having high or low concentrations of progesterone. Cows were lutectomized and then supplemented with progesterone (150 mg every 12 h) to maintain pregnancy. After lutectomy, area under the curve for concentration of progesterone was greater in

cows previously classified as high progesterone than low progesterone. In the removed CL, gene expression for endothelin and prostaglandin systems between cows designated as high or low were not different. These data support a greater involvement in progesterone inactivation than in progesterone secretion for controlling peripheral concentrations (Rhinehart et al., 2009).

Nutrition affects reproductive efficiency in many species and can impact inactivation of progesterone. In sheep, an increase in dry matter intake (DMI) during early pregnancy resulted in an increased occurrence of embryonic loss (Parr et al., 1987). An increase in DMI at time of artificial insemination resulted in decreased embryo survival in beef cattle (Dunne et al., 1999) and swine (den Hartog and van Kempen, 1980). Dry matter intake is highly correlated with milk production ($r = 0.88$) in dairy cattle (Harrison et al., 1990). Sangsritavong et al. (2002) found that greater feed intake increased hepatic blood flow and inactivation of progesterone and estradiol-17β. Hepatic blood flow was greater in lactating cows (1,561 ± 57 L/h) compared to non-lactating cows (747 ± 47 L/h). In lactating cows, hepatic blood flow was greater in cows fed 2.2 times maintenance (1,984 ± 62 L/h; 15.2 kg dry matter) than in the unfed (1547 ± 57 L/h), 0.5 (1,685 ± 60 L/h; 3.54 kg dry matter), and 1.5 (1,817 ± 56 L/h; 10.62 kg dry matter) times maintenance groups at 4 h post-feeding (Sangsritavong et al., 2002). In a study comparing ad libitum verses restricted access to pastures, Rabiee et al. (2001) found an increase in progesterone metabolites in the feces of dairy cows allowed to graze ad libitum. The increase in progesterone metabolites in the feces also corresponded with decreased circulating progesterone in the ad libitum fed (1.08 ± 0.03 ng/ml) cows compared to the restricted (1.71 ± 0.04 ng/ml) diet. The decrease in reproductive

efficiency observed in previous studies might be a result of an increase in hepatic blood flow and a decrease in concentration of peripheral progesterone due to an increase in progesterone inactivation.

Dry matter intake is not the only factor that affects steroid hormone inactivation. In a study by Smith et al. (2006), ewes were allocated to treatment groups and orally gavaged with sodium acetate or sodium propionate. Ewes gavaged with sodium propionate had increased concentrations of insulin and increased concentrations of progesterone compared to ewes gavaged with sodium acetate. In a similar study by Lemley et al. (2008), ewes were fed three times a day and supplemented with sodium acetate or sodium propionate. This study also found an increase in concentrations of insulin after feeding. Furthermore, hepatic CYP3A and CYP2C activity were decreased in ewes fed sodium propionate compared to sodium acetate. In another study by Lemley et al. (2010), dairy cows were fed the same amount of dry matter with one group receiving a high cornstarch diet and the other a high fiber diet that was isoenergetic and isonitrogenous. Cows fed a high cornstarch diet had increased secretion of insulin and decreased hepatic activity of CYP2C and CYP3A (Lemley et al., 2010). They concluded that by feeding diets that would stimulate insulin secretion, it could be possible to manipulate progesterone inactivation in cows during times of increased energy requirements, dry matter intake, and milk production (Lemley et al., 2010). More research is needed to explore the effects of other factors that might influence progesterone clearance, as well as therapeutics that could potentially decrease the inactivation rate of progesterone in dairy cattle. This research may lead to improved fertility and decreased early embryonic mortality in dairy cattle.

Corpus Luteum

After ovulation, luteal tissue begins to form rapidly from the walls of the ruptured follicle. Cells of the theca interna and granulosal cells begin to undergo dramatic changes in a process known as luteinization. Luteinization is the transformation of the ovulatory follicle cells into luteal cells. The CL consists of several different types of cells including small and large luteal cells which are steroidogenic, as well as nonsteroidogenic cells, such as endothelial cells and fibroblasts (Niswender, 2000). Large luteal cells originate from the granulosal cells and small luteal cells originate from theca interna cells.

Many angiogenic and vasoactive factors play an important part in the development of the early CL, including vascular endothelial growth factor, fibroblast growth factor, and nitric oxide (Miyamoto et al., 2009). By the mid-luteal phase, based on size, the CL has the greatest blood flow of any organ due to the complex vascular network of capillary beds (Reynolds et al., 2000). Most steroidogenic luteal cells within the CL are adjacent to one or more capillaries. Due to the vascularity and rapid growth and formation, the CL has been utilized as a model for tumor research (Reynolds et al., 2000).

Human Chorionic Gonadotropin

Human chorionic gonadotropin (hCG) is a member of the glycoprotein hormone family, which also includes luteinizing hormone (LH), follicle stimulating hormone, and thyroid stimulating hormone (Stenman et al., 2006). Glycoprotein hormones consist of two different subunits, α and β, with the amino acid sequence in the α-subunits being common to all glycoproteins and the β-subunits controlling biological activity. The β-subunits of hCG and LH share approximately 80% of the same amino acid sequence,

resulting in hCG having LH-like activity (Pierce and Parsons, 1981; Stenman et al., 2006).

In humans, the action of hCG is mediated through the LH/hCG receptor which is responsible for maintaining the production of progesterone from the CL during early pregnancy (Stenman et al., 2006). Due to the luteinizing activity of hCG on the ovaries, its use in cattle has resulted in many different ovarian responses including: extended life span of the CL (Sianangama and Rajamahendran, 1992), induced ovulation throughout the estrous cycle (Price and Webb, 1989), and the formation of accessory CL (Sianangama and Rajamahendran, 1992; Santos et al., 2001; Stevenson and Pulley, 2012).

The treatment of hCG early in the estrous cycle and post-insemination has been examined by several research groups. Walton et al. (1990) independently administered progesterone via a progesterone-releasing intravaginal device, an injection of progestin, or an injection of hCG 5 d post-insemination. This research group found a sustained increase in concentrations of progesterone in milk from d 14 to 20 with the use of hCG but conception rates were not different among the treatment groups despite the increase in progesterone later in the estrous cycle. Furthermore, the estrous cycle length was not extended in the hCG group, making hCG an acceptable technique for supplementation of progesterone. The effectiveness of hCG to cause a spontaneous ovulation and induce an accessory CL when administered is approximately 70% (Wallace et al., 2011; Stevenson and Pulley, 2012).

Twinning and Double Ovulation

Double ovulation is the release of two oocytes during the same estrous cycle in a monovular species. In some instances, double ovulation will result in the event of twinning. The occurrence of twinning is between 2.4 and 5.8% in dairy cattle (Kinsel et al., 1998; Wiltbank et al., 2000), with 93% of twins being non-identical (Silva Del Rio et al., 2006), indicating they were a result of double ovulation. The incidence of double ovulation, however, is more common than twinning because of embryonic mortality and is between 5 and 28% (Fricke and Wiltbank, 1999; Lopez-Gatius et al., 2005; Mann et al., 2007). Factors that are linked to twinning and double ovulation rates include milk yield, parity, genetics, use of antibiotics or hormone therapy, ovarian cysts, and days open (Kinsel et al., 1998; Wiltbank et al., 2000; Lopez-Gatius et al., 2005). Of these factors, milk yield is believed to be the largest contributor to twinning (Kinsel et al., 1998). Fricke and Wiltbank (1999) found a 3-fold increase in twinning between cows producing greater than 40 kg and those producing less than 40 kg of milk. In a similar study, cows producing over 50 kg of milk per day had a 51.6% double ovulation rate. As milk production decreased, so did the rate of double ovulation (Lopez et al., 2005a). Lopez et al. (2005b) found that a transient increase in concentrations of follicle stimulating hormone 24 h before the deviation of the dominant follicle resulted in an increased occurrence of multiple ovulations.

In the event of twinning, cows are at an increased risk of dystocia, stillbirth, retained placenta, ketosis, milk fever, and displaced abomasum (Nielen et al., 1989). Involuntary culling rates are also increased for cows calving twins. Beerepoot et al. (1992) estimated the total losses of twinning to be around $171 per twinning incident,

therefore, twinning is considered to be undesirable in dairy cattle. Furthermore, calf mortality is greater in twin births compared to singleton births, 28.2 vs 7.2 % respectively (Silva Del Rio et al., 2006).

Lopez et al. (2005b) reported that circulating concentrations of progesterone around the time of follicle selection were decreased in animals that had a double ovulation and also had an increase in follicle stimulating hormone. Cows with a double ovulation had an increase in total luteal volume and decreased peripheral concentrations of progesterone 7 d after ovulation compared to single ovulation cows (Lopez et al., 2005a). In contrast, Mann et al. (2007) found that cows with a double ovulation had similar luteal weights and peripheral concentrations of progesterone when compared to cows with a single ovulation on d 5 and 8 post-ovulation. Double ovulation could cause changes in the CL thus, altering concentrations of progesterone and influencing reproductive efficiency in cattle.

Ultrasonography

In the early 1980s, the utilization of ultrasonography as a tool to view internal organs, especially the reproductive tract, became available for use in cattle. Ultrasonography is the use of high frequency sound waves to produce images of internal structures. Sound waves are sent out and return to a probe after bouncing off an object, similar to sonar used on ships to map the ocean floor. The density of the tissue determines how that object appears, ranging in color from black to white including various shades of gray. For example, liquid such as follicular fluid does not reflect sound, therefore it appears black on the screen. More dense tissue, such as bone, will appear white with intermediate density tissue (reproductive tissue) appearing in shades of gray

(Pierson et al., 1988). The most common form of early ultrasonography was the utilization of the 2-dimensianal B-mode (brightness) imaging.

More recent advances in ultrasonography have allowed for the use of color Doppler ultrasonography. The color Doppler technique uses a color-coded overlay of a B-mode ultrasound image to depict the changing blood velocities within vessels by estimating the Doppler mean frequency shift in the echoes of red blood cells as they move towards or away from the probe (Martinoli et al., 1998; Ginther, 2007).

A variation of color Doppler is power flow Doppler ultrasonography. Power flow imaging displays a similar color-coded overlay of a B-mode image using variations in shades of a single color. The intensity or shade in color is generated by determining the power of the Doppler signal for each pixel instead of the Doppler frequency shift (Ginther, 2007). Since power flow Doppler detects blood flow intensity instead of velocity, it works independent of the angle of sound waves and blood flow and has several advantages over color Doppler ultrasonography including improved sensitivity and the ability to characterize flow in vessels too small or with decreased blood flow for conventional color Doppler to detect (Martinoli et al., 1998; Ginther and Utt, 2004; Ginther, 2007). With the use of power flow ultrasonography, blood perfusion of a tissue can now be characterized and quantified (Ginther, 2007). Using color pixel analysis of three different images recorded from the same examination period of a CL in mares, Bollwein et al. (2002) found an interclass correlation coefficient of 0.90, showing that color pixel analysis is a repeatable and effective way to determine luteal blood perfusion.

Due to the high vascularity of the CL, power Doppler ultrasonography can be used to detect CL blood perfusion. Herzog et al. (2010) assessed the reliability of luteal

blood perfusion, via power Doppler ultrasonography, in conjunction with luteal size and circulating progesterone in cattle. They found a strong, positive correlation between luteal blood perfusion and plasma progesterone concentrations. These data support the findings of Acosta et al. (2002), which emphasized that luteal blood supply is essential for the secretion of progesterone.

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CHAPTER II

EFFECT OF DOUBLE OVULATION ON CORPUS LUTEUM BLOOD PERFUSION, PROGESTERONE, AND HEPATIC STEROID INACTIVATING ENZYMES

Introduction

Steady declines in reproductive efficiency in dairy cattle have been seen over the past 60 years including decreased pregnancy rates and increased embryonic mortality. Decreased concentrations of progesterone due to increased catabolism, decreased secretion, or both could be responsible (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 glucuronsyltransferase (UGT). Cytochrome P450 2C (CYP2C) is the largest contributor to progesterone inactivation in the liver followed by AKR1C and CYP3A, respectively (Lemley and Wilson, 2010). Regulation of these enzymes seems to be dependent on the rate of hepatic blood flow. Sangsritavong et al. (2002) found that increased feed intake increased hepatic blood flow and inactivation of progesterone.

Double ovulation is common in dairy cattle but can lead to the undesirable occurrence of twinning. Silva Del Rio et al. (2006) found that 93% of twins in dairy cattle are non-identical meaning that the majority of twins are the result of a double ovulation.

Cows carrying a twin pregnancy are 5.4 times more likely to experience pregnancy loss than singleton pregnancies (Lopez-Gatius et al., 2004). The physiological mechanism for this difference in pregnancy loss still eludes researchers.

Advances in technology for ultrasonography have allowed for the monitoring and quantification of blood perfusion of the 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 concentrations of progesterone in cattle. They found a strong, 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 at the Bearden Dairy Research Center (Starkville, MS) were housed in a free stall barn and fed, ad libitum, a total mixed ration (TMR) formulated to meet or exceed dietary requirements of lactating cows (NRC, 2001). Average DIM was 289 ± 60 (range of 163 to 399) and average milk production was 32.4 ± 9.7 kg/d. From mid-July to early November, cows had their estrous cycle

synchronized using Ovsynch (Pursley et al., 1995), with d 0 being the day of the second injection of gonadotropin releasing hormone. 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 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 Ovsynch and followed the same process as previously stated.

On d 10, cows that had received hCG were required to have two CL and those that failed to produce one accessory CL in response to the 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 one accessory CL were re-submitted to Ovsynch and subsequent treatments and evaluations.

From d 10 to 18 or until CL regression, ovaries were mapped using transrectal ultrasonography. The diameter of each CL was measured and volume calculated using the previously published method (Vasconcelos et al., 2001). Blood perfusion of the CL was evaluated using the power flow option of the ultrasound machine and two still images and one video were recorded for each CL for later analysis. Blood samples were collected daily from d 10 to 18 by venipuncture in a spray-coated K2 EDTA tube (Becton, Dickinson, and Company, Franklin Lakes, NJ) and immediately placed on ice.

Within 2 h, tubes were centrifuged at 2,000 x g at 4°C for 20 minutes and plasma was frozen at -20°C until later analysis.

On d 13, a liver biopsy was performed on the right side of the cow at the 10th intercostal space following the previously published methods of Lemley et al. (2010). Hair was removed with a clipper and an ultrasound scan of the liver was performed to locate and avoid large hepatic vessels during the biopsy. The area of the 10th intercostal space was scrubbed three times with betadine (Purdue Products L. P., Stamford, CT) and a local anesthetic (approximately 10 mL of 2% lidocaine hydrochloride; MWI, Bosie, ID) was administered. After a short cessation, the skin was punctured with a scalpel and the liver sample was collected using a biopsy needle machined at Mississippi State University Agricultural and Biological Engineering Department (Mississippi State, MS) 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 or after healed. Cows were observed and body temperatures were taken to monitor for complications. Body weights were recorded prior to 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 prostaglandin F2α (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 x g for 10 min at 4°C. The supernatant was aliquoted into 2 microcentrifuge tubes and stored at -80°C until enzyme assays were conducted. The protein concentration of the supernatant was determined by a Coommasie Plus (Bradford) protein assay following the manufacture's protocol (Thermo Scientific, Rockford, IL).

Cytochrome P450 Enzymes (CYP)

Assay kits for CYP1A, CYP3A, and CYP2C and NADPH regeneration system were used (Promega Corporation, Madison, WI) and completed following the manufacturer's protocol. Previous validation was performed by Hart et al. (unpublished data). Briefly, reconstitution buffer was added to luciferin detection reagent. Luciferin CEE (CYP1A), luciferin IPA (CYP3A), and luciferin H (CYP2C) were diluted in KPO4 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 pre-incubated 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 the plate was incubated for 30 min (CYP1A) and CYP2C) at 37°C or 10 min (CYP3A) at room temperature. After the incubation, 50 µL of luciferin detection reagent was added to each well, plates were covered to protect from light, and incubated for an additional 20 min at room

temperature. Plates were then placed into a Promega Multi-Plus (Madison, WI) plate reader and read with luminescence detection mode.

Uridine 5'-diphosphate glucuronsyltransferase (UGT)

The UGT assay kit was used (Promega Corporation) and performed following the manufacturer's instructions with minor modifications. Previous validation of this modified procedure was performed by Hart et al. (unpublished data). Briefly, 10 µL of uridine diphosphoglucoronic acid (UDPGA) was added to half the plates to act as reaction wells and 10 µL of distilled water was added to the other half as control wells. Reaction mixture of UGT (13 µL) and 28 µg of liver protein were then added to all the wells. The plate was then pre-incubated for 10 min at 37°C. After the incubation, 40 µL of detection reagent was added to each of the wells followed by an incubation period of 20 min at room temperature. During the incubation period the plate was covered to protect from light. The plate was then read using a Promega Multi-Plus plate reader and read with luminescence detection mode.

Aldo-keto reductase 1C (AKR1C)

The AKR1C activity was determined following the previously published methods of Lemley and Wilson (2010). Briefly, AKR1C was determined using the specific substrate, 1-acenapthenol (Pfaltz & Bauer, Waterbury, CT). Enzymatic reactions contained 150 µg of cytoplasmic protein, 250 µM 1-acenapthenol, and 500 µM NADP. The 1-acenapthenol 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 L/ mol x cm) was used to calculate the rate of reduced NADP in pmol per min per mg of protein.

Radioimmunoassay

Concentrations of progesterone were determined by RIA using a Coat-A-Count (Siemens Healthcare Diagnostics Inc., Los Angeles, CA) kit and following the manufacture's protocol. Briefly, standards or 100 µL of plasma were added, in duplicate, to the coated tubes. Next, 1 mL of I125 progesterone tracer was added to each tube. Following the 3 h incubation, tubes were aspirated, allowed to drain thoroughly, and activity was assessed with a gamma counter (Packard Instrument Company, Meriden, CT). The intra-assay 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, Maryland) for integrated density to quantify total pixels of blood perfusion. Images and videos were visually scored by two 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

The concentration of progesterone, total luteal volume, and blood perfusion of the CL were analyzed using repeated measurements in the MIXED procedure of SAS (SAS software version 9.3, SAS Institute Inc., Cary, NC) with autoregressive-1 as the covariate

structure. Cow was considered a random variable and milk production, DIM and body weight were considered covariates. Treatment effect for hepatic enzymes was analyzed using the MIXED procedure of SAS. Means were separated using the PDIFF option of the LSMEANS statement. Pearson correlation coefficients were determined using the CORR procedure of SAS. Least-square means and pooled standard error are reported. Statistical significance was declared at $P < 0.05$.

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) or cows with 2 CL (7.73 ± 0.84 ng/mL). The interaction ($P = 0.02$) between treatment and day for total luteum volume is shown in Figure 1. On d 10 to 18, total luteal volumes were not different between treatment groups within a given day.

Total integrated densities ($P = 0.001$) were greater in cows with 2 CL (7,781.44 ± 758.06 pixels) than cows with 1 CL (4,399.69 ± 638.95 pixels). Similarly, blood perfusion scores ($P < 0.001$) were greater in cows with 2 CL (6.96 ± 0.32) than cows with 1 CL (3.82 ± 0.27) for images visually scored. The treatment by day interaction ($P = 0.02$) is shown in 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 on d 18 ($P = 0.10$).

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 ($P < 0.02$). 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 ($P = 0.003$).

The activity of hepatic steroid inactivating enzymes CYP1A, CYP3A, CYP2C, AKR1C, and UGT (Table 1) were not different ($P > 0.18$) based on milligram of protein and per kilogram of body weight on d 13 between cows with 1 CL and cows with 2 CL.

Progesterone tended to be positively correlated with total luteal volume ($P = 0.10$; Table 2). Progesterone tended to be negatively correlated with milk production ($P = 0.06$; Table 2). Image and 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 score 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 to luteal volume, progesterone, or CL blood perfusion.

Discussion

Expected synchronization rates in dairy cattle using Ovsynch are approximately 87% (Vasconcelos et al., 1999). In the current study, response rates using Ovsynch were well below expected outcomes (63.3 vs 87%). The observed response in the current study to hCG to induce an accessory CL (36%) was also less than expected. Previous reports

using hCG to cause a spontaneous ovulation and induce an accessory CL have been approximately 70% (Wallace et al., 2011; Stevenson and Pulley, 2012). The poor response 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 to 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 non-identical (Silva Del Rio et al., 2006). The incidence of double ovulation, however, is more likely than twinning and is reported to be between 5 and 28% (Fricke and Wiltbank, 1999; Lopez-Gatius et al., 2005; Mann et al., 2007). Beerepoot et al. (1992) estimated that twin births cost producers, on average, $171 more per incidence when compared to singleton births. Understanding the mechanisms and physiological changes involved with double ovulation could potentially help decrease the occurrences of twinning in dairy cattle, thus, making a positive economical impact for producers.

Similar to results in this current study, Mann et al. (2007) determined that dairy cows, with a double ovulation 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 than cows with a single ovulation. In beef cattle genetically selected for twining, Echternkamp et al. (2009) found that cows with a double ovulation had greater total luteal volume and concentrations of progesterone compared to cows with a single ovulation. Interestingly, Sanjabi ewes with a double ovulation had decreased luteal volume but increased concentrations of progesterone compared to ewes with a single ovulation (Shabankareh et al., 2009). Though the size of the hCG-induced ovulatory follicle was not measured, it

would be expected that the size of the follicle 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 a difference in the total luteal volume between cows with 1 CL and cows with 2 CL in the current study.

The use of luteal volume and luteal blood flow (perfusion) can be used as an indicator of luteal function and concentrations of peripheral progesterone (Herzog et al., 2010). Herzog et al. (2010) found a high, positive correlation between luteal volume and concentrations of progesterone ($R = 0.69$; $P < 0.001$) as well as a high, positive correlation between luteal blood flow and concentrations of progesterone ($R = 0.71$; $P < 0.001$). In the current study, total luteal volume only tended to be positively correlated with concentrations of progesterone. Furthermore, CL blood perfusion was not correlated to concentrations of progesterone. Despite the increase in perfusion observed in cows with 2 CL in this study, the total luteal volume was the same.

The system of visually scoring CL blood perfusion is a fairly novel idea, while the use of imaging software to determine pixel quantity has been documented (Ginther and Utt, 2004; Herzog et al., 2010). In the current study, there was no difference between perfusion scores obtained from images versus videos, therefore either images or videos can be effective sources for visual scoring CL blood perfusion. The high, 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, it appears that hepatic enzymes were not altered by an induced double ovulation, but it is important to note that there was also no difference in concentrations of progesterone between treatments. Previous studies have shown that an increase in hepatic blood flow increases the inactivation of steroid hormones, such as progesterone, thus decreasing the peripheral concentrations (Sangsritavong et al., 2002). Rhinehart et al. (2009) showed a larger role of inactivation than secretion of progesterone in controlling peripheral concentrations.

Conclusion

Overall, the increased blood perfusion of CL in cows with 2 CL did not correspond to peripheral concentrations of progesterone or clearance as measured by hepatic enzyme activity, perhaps indicating that a double ovulation does not impact 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.

Table 1 Hepatic cytochrome P450 (CYP), aldo-keto reductase (AKR), and uridine diphosphate glucuronsyltransferase (UGT) activity

Enzyme*	Control	hCG†	SE	*P*-value
CYP1A				
RLU/min/mg of protein x10^4	44.26	35.09	4.74	0.18
RLU/min/kg bw	20.46	15.95	2.41	0.19
CYP3A				
RLU/min/mg of protein x10^5	15.76	14.80	1.55	0.66
RLU/min/kg bw	73.23	67.48	8.42	0.63
CYP2C				
RLU/min/mg of protein x10^3	4.58	4.60	0.39	0.98
RLU/min/kg bw	0.21	0.21	0.02	0.90
AKR1C				
RLU/min/mg of protein	48.39	43.39	2.77	0.21
RLU/min/kg bw x10^3	24.49	20.69	2.00	0.19
UGT				
RLU/min/mg of protein x10^4	98.45	95.33	11.30	0.84
RLU/min/kg bw x10^8	6.49	6.12	0.81	0.74

* Relative light units (RLU)
† Human chorionic gonadotropin (hCG)

Table 2 Pearson correlations (r) between measurments on d 13

	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*

* $P < 0.05$
† $P > 0.05$ but ≤ 0.10

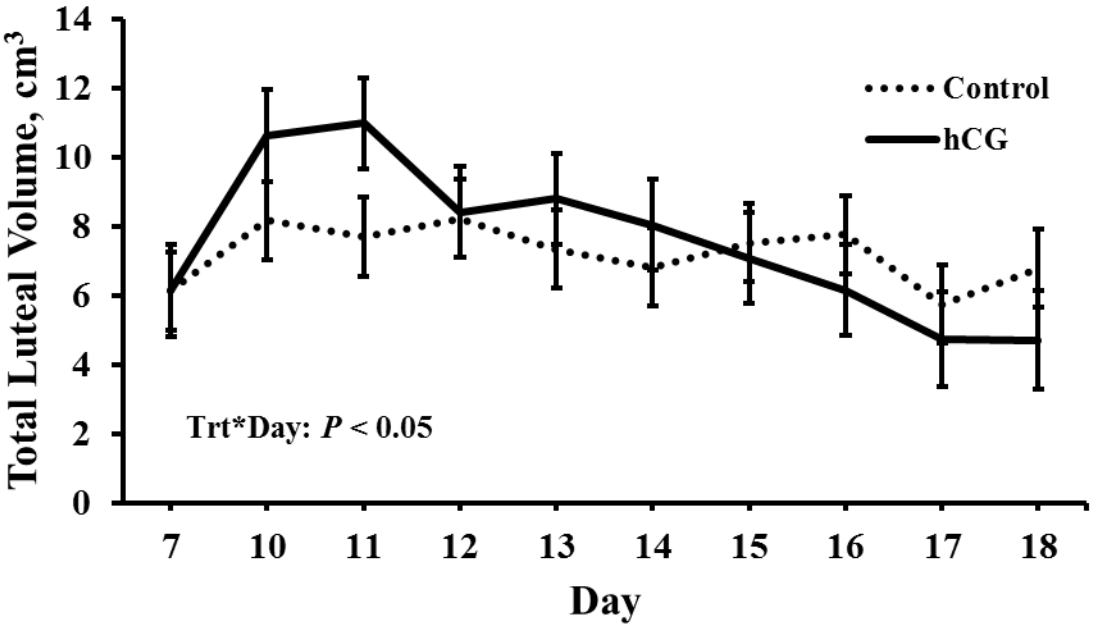

Figure 1 Total luteal volume between cows with 1 or 2 corpora lutea

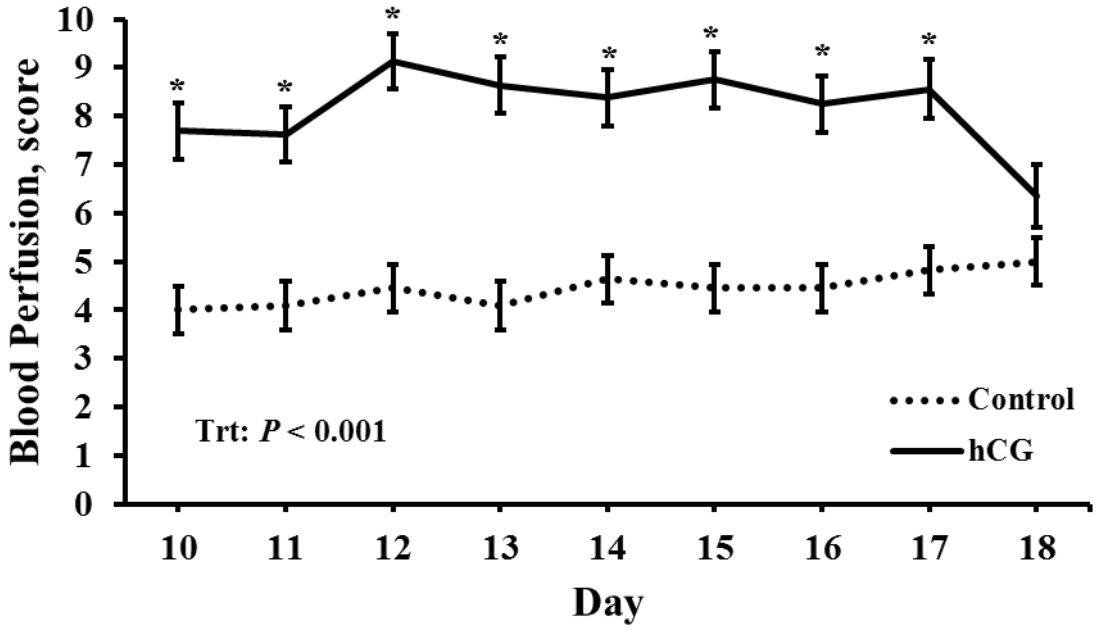

Figure 2 Visual characterization of total luteal blood perfusion from analysis of images

* $P < 0.05$

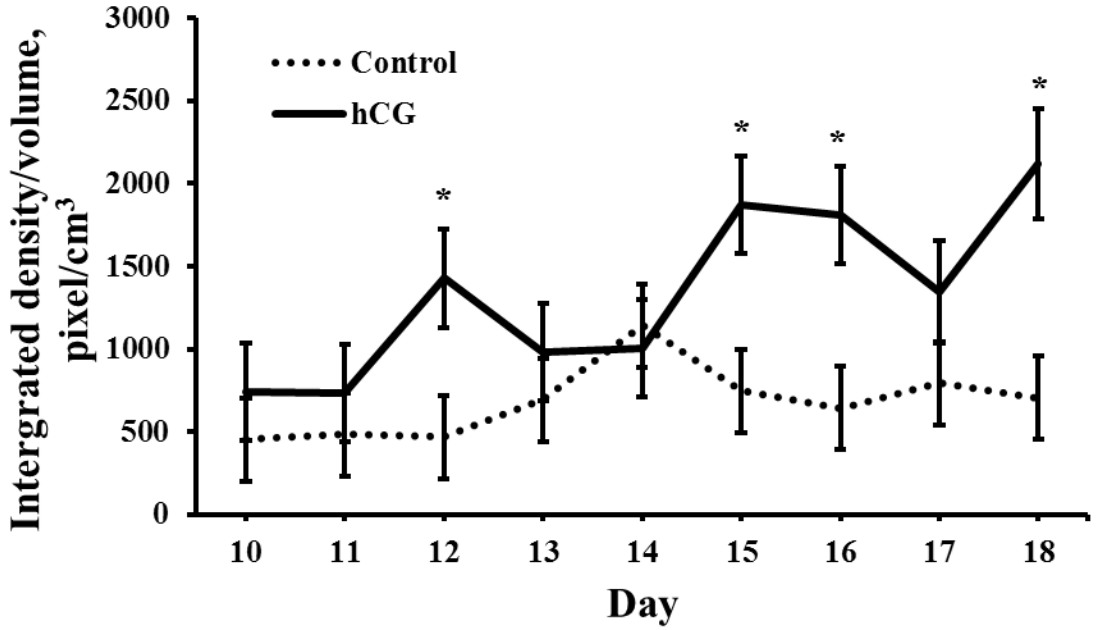

Figure 3 Volume adjusted integrated density perfusion

* $P < 0.05$

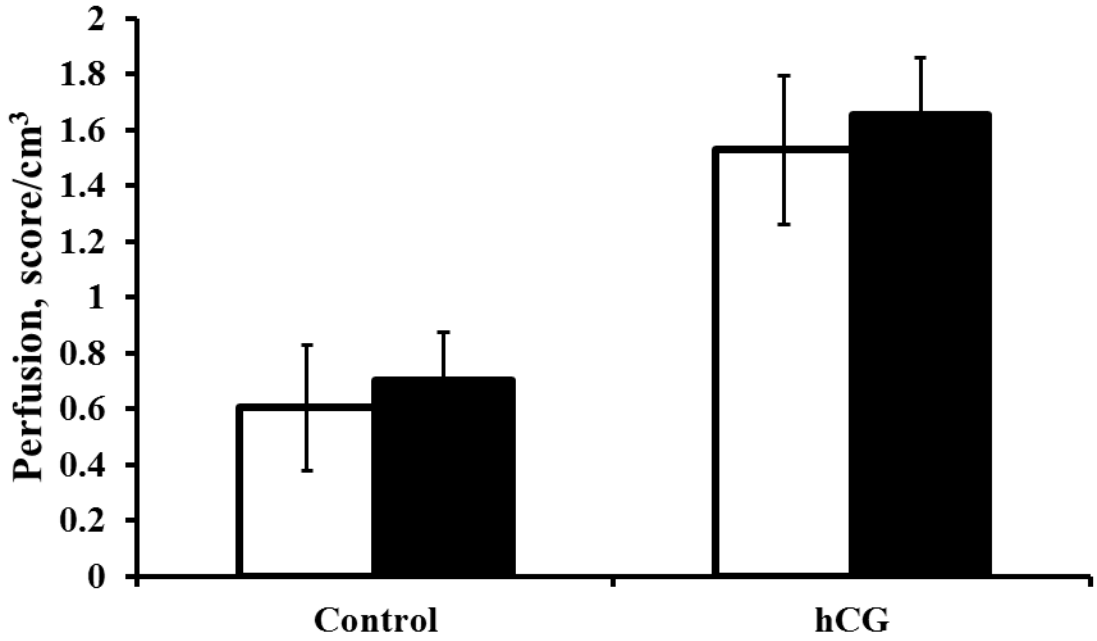

Figure 4 Volume adjusted perfusion for images and videos

Image perfusion scores are shown as a white bar and video perfusion scores are shown as a black bar

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