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Incorporating follicle stimulating hormone to stimulate multiple corpora lutea for embryo transfer in beef cattle

Bryan Carter

Mississippi State University, bryancarter1992@hotmail.com

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Incorporating follicle stimulating hormone to stimulate multiple corpora lutea for embryo

transfer in beef cattle

By

Bryan Carter

Jamie Larson (Major Professor/Graduate Coordinator)
Caleb O. Lemley
Brandi B. Karisch
Trent Smith
Scott T. Willard (Dean, College of Agriculture and Life Sciences)

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Name: Bryan Carter

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Assisted reproductive technologies, such as estrus synchronization and embryo transfer, can aid producers in improving genetics by increasing the number of progeny produced from elite females. The success of embryo transfer is dependent on a viable, competent embryo and a recipient with a receptive uterine environment. Follicular development and luteinization are pertinent for the recipient to establish a functional corpus luteum (CL) that can produce adequate concentrations of progesterone (P4) and provide a uterine environment conducive for the establishment of pregnancy. The objective of this study was to determine if exogenous follicle stimulating hormone (FSH), would increase the number of CL, size and blood perfusion of the largest CL, as well as circulatory concentrations of P4.

ACKNOWLEDGEMENTS

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CHAPTER I

LITERATURE REVIEW

Introduction

Embryo transfer is a reproductive technique that can be used to increase the number of

progeny produced from superior females. Cows and heifers are physiologically restricted to one

pregnancy per year which decreases the female's impact on genetic improvement compared to

males. Embryo transfer allows surrogate dams (recipients) to gestate the embryos from

genetically superior females, translating to increased female impact on genetic improvement.

Thus, embryo transfer has become a desirable application in seedstock cattle operations.

However, there are many factors that must coincide for successful establishment of pregnancy

after the transfer of an embryo. The following literature review discusses the current knowledge

about beef cattle reproduction, specifically reproductive anatomy, endocrine control, estrus

synchronization protocols, and the requirements for successful establishment and maintenance of

pregnancy.

Estrous Cycle

Cattle typically have a 21-day estrous cycle, but it can vary between 17 and 24 days

(Armstrong and Hansel, 1958). Standing estrus is typically referred to as day 0 of the estrous

cycle and the cycle ends just prior to the onset of the subsequent estrus. The estrous cycle is

divided into two phases, the follicular phase and luteal phase (Rodriguez-Martinez et al., 1986).

<u>Follicular Phase</u>

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The follicular phase can be further divided into two stages: proestrus and estrus. Proestrus begins after luteolysis and ends just prior to estrus. A cohort of follicles is initially recruited by follicle stimulating hormone (FSH), then selected by luteinizing hormone (LH) to reach ovulatory competence (Knox, 2010). The dominant follicle undergoes a final growth and maturation period secreting localized inhibin causing subordinate follicles to undergo atresia (Dean, 2009). Estradiol produced by the dominant follicle increases until a threshold concentration in the hypothalamus is reached, initiating a cascade of events leading to ovulation (Chenault et al., 1974).

Cattle exhibiting a longer proestrus period have improved embryo development, luteal function, and pregnancy rates (Burke et al., 2001; Mussard et al., 2003; 2007; Bridges et al., 2010 Pohler et al., 2012). Bridges et al. (2010) evaluated the effects of the length of proestrus and described cattle as having a long proestrus (LPE, 2.25 days) or a short proestrus (SPE, 1.25 days). Bridges et al. (2010) found that cattle with a LPE period had increased circulatory concentrations of estrogen (E2) at estrus (12.7 ± 1.1 vs 9.1 ± 0.7 pg/mL, $P < 0.05$), increased concentrations of P4 during the mid-luteal phase (4.8 ± 0.2 vs 3.1 ± 0.2 ng/mL, $P < 0.05$), and improved pregnancy rates (73.0 vs 14.3%, $P < 0.01$) compared to cattle with a SPE period. Additionally, the number of cows having a shorter duration of luteal phase in the subsequent estrous cycle were greater in cattle with a SPE period compared to cattle with a LPE period (81.5 vs 35.0%, $P < 0.05$) (Bridges et al., 2010). These results indicate that cattle with a longer proestrus period have increased ovulatory concentrations of E2 and increased mid-luteal phase concentrations of P4. Increased concentration of mid-luteal phase P4 could a have greater ability to inhibit prostaglandin F2α (PGF2α) pulsatile secretion from the uterine endometrium and delay luteolysis, thus resulting in a longer subsequent luteal duration, and increased pregnancy rates.

Estrus is classified by increased concentrations of E2 produced by the dominant follicle. Increasing E2 causes females to display secondary sex characteristics, increase uterine tone, increase cervical mucosal secretions, and modulate blood flow to the reproductive tract (Rodriguez- Martinez et al., 1986). According to Chenault et al. (1974) E2 concentrations peak 36 hours prior to ovulation. Abreu (2014) evaluated estrus expression based on follicle maturity and found that cows with mature follicles had increased expression of estrus after $PGF_{2\alpha}$ compared to cows with young follicles (76.6 vs 48.3%, $P < 0.01$). These results from Abreu (2011) coincide with the results found by Bridges et al. (2010), concluding that cows with a longer proestrus period and a larger ovulatory follicle have increased expression of estrus.

Perry et al. (2007) found that heifers exhibiting standing estrus had increased ($P < 0.01$) follicle diameter at timed artificial insemination (TAI; 12.2 ± 0.2 vs 11.1 ± 0.3 mm), increased concentrations of E2 at TAI (9.9 ± 0.6 vs 6.6 ± 0.7 pg/mL), and increased pregnancy rates (63 vs 20%) compared to those that did not exhibit standing estrus at TAI.

Madureira et al. (2021) evaluated the duration of estrus expression and cows with a longer duration of estrus expression had increased concentrations of P4 4 days prior to TAI ($P < 0.05$), and decreased concentrations of P4 at TAI (4.6 ± 0.2 vs 3.6 ± 0.2 ng/mL, $P < 0.05$). Additionally, Madureira et al. (2021) found that cows with a shorter duration of estrus expression had decreased concentrations of E_2 at estrus ($P < 0.01$). These results indicate that cows with increased concentrations of P4 during follicular development had increased expression of estrus. This could be explained by the presence of a CL capable of producing increased circulatory concentrations of P4 prior to $PGF_{2\alpha}$ administration followed by successful luteolysis after administration of $PGF_{2\alpha}$.

Therefore, a functional CL containing prostaglandin receptors can respond to administration of PGF2α and undergo complete luteolysis, leading to decreased concentrations of P4 at TAI. Cattle without a functional CL or containing an immature CL at administration of PGF2α may not respond to PGF2α, thus leading to incomplete or delayed luteolysis with increased concentrations of P4 at TAI. Increased concentrations of P4 at TAI have negative feedback on GnRH secretion in the hypothalamus leading to decreased secretory concentrations of E2 from the dominant follicle that negatively affects follicle maturity and expression of estrus (Madureira et al., 2021).

Overall, these results indicate that the expression of estrus is positively correlated with diameter of the ovulatory follicle diameter, concentration of E2 at estrus and pregnancy rates as well as negatively correlated with concentration of P4 concentrations at estrus.

Luteal Phase

The luteal phase is divided into two stages: metestrus and diestrus (Wildt et al., 1981). Metestrus follows estrus and contains the corpus hemorrhagicum or "bloody body" which is the early formation of the CL structure (Wildt et al., 1981). Around the time of ovulation granulosa and theca interna cells begin to luteinize and form large and small and luteal cells, respectively (Richards et al., 2018; Abedel-Majed et al., 2019). The transformation from follicular to luteal cells is governed by LH. Small and large luteal cells begin to mix uniformly, creating a vascularized network within the CL (Richards et al., 2018). The total number of granulosa cells donated by the ovulatory follicle determines the number of large luteal cells in the newly formed CL (Richards et al., 2018). Large luteal cells undergo hypertrophy, while small luteal cells undergo hyperplasia as the CL develops, although both are steroidogenic (Abedel-Majed et al., 2019). This is significant because large luteal cells cannot undergo mitosis to produce more large

luteal cells, therefore the number of granulosa cells donated by the follicle determines the number of large luteal cells and size of the CL.

Diestrus begins when the CL becomes "mature" around day 5 post-estrus and lasts until luteolysis occurs (Bazer et al., 2012). During this period, the CL is responsive to $PGF_{2\alpha}$ and is susceptible to undergoing luteolysis (Bogan et al., 2008) which occurs between days 15 and 18 of the estrous cycle (Pugliesi et al., 2016). Luteolysis is caused by a pulsatile release of $PGF2\alpha$ from the uterine endometrium (McCracken et al., 1999) causing luteal tissue of the CL to undergo irreversible degeneration, leading to decreased concentrations of P4 (Okuda and Sakumoto, 2003).

Concentration of P4 during metestrus rapidly increases (Ajayi and Akhigbe, 2020). However, concentration of P4 is greatest during diestrus due to a fully functional CL (Baerwald et al., 2005). Stabenfeldt et al. (1969) evaluated concentrations of P4 by collecting blood samples every day from cows (n = 6) for 7 consecutive estrous cycles. Concentration of P4 increased rapidly from day 3 to day 8 and then increased at a much slower rate from day 8 to day 17. Concentrations of P4 ranged from less than 0.5 ng/mL during the follicular phase and peaked at an average of 6.6 ng/mL during the luteal phase ranging from day 11 to day 20. Following luteolysis, concentrations of P4 decreased more than 50% from the within 24 hours. After the decline in concentrations of P4, return to estrus ranged from 1 to 5 days.

Estrus Synchronization

Many estrus synchronization protocols include gonadotropin releasing hormone (GnRH) to induce ovulation and initiate a new follicular wave along with a controlled internal drug release (CIDR) infused with P4. This device is placed in the vagina and P4 is absorbed through

the vaginal wall and acts through a negative feedback mechanism to prevent spontaneous

ovulation. At CIDR removal, $PGF_{2\alpha}$ is administered to induce luteolysis which is required for

reducing concentration of P4. After the CL undergoes complete luteolysis, follicular maturation

is allowed to occur and the GnRH surge can initiate ovulation (Holesh et al., 2022). A TAI

protocol uses exogenous GnRH at the time of insemination to induce ovulation 28-30 hours later

(Stevenson, 2004).

Estrus synchronization protocols are used in embryo transfer programs to synchronize the

timing of ovulation within a herd. Most often, an embryo technician is scheduled to transfer

embryos on a specific day. Therefore, to maximize pregnancy rates, the recipient must express

estrus $\pm$ 24 hours from the expected time of estrus so her uterine environment matches that of the

donor (Wright, 1981).

Spell et al. (2001) evaluated the efficacy of estrus synchronization in cows (n = 763)

using the two-shot prostaglandin protocol where two doses of PGF2α were administered 11 days

apart. Estrus detection was performed for 5 days following the last injection of PGF2α. Of the

total cows subjected to estrus synchronization, 526 (68.9%) cows displayed estrus and were

presented for embryo transfer. Of the 526 cows displaying standing estrus, 448 (85.2%) cows

presented an adequate CL structure and received an embryo. In all, 58.7% (448/763) of the total

cows subjected to estrus synchronization were eligible to receive an embryo at day 7 post-estrus.

Bonacker et al. (2020) compared estrus synchronization protocols in beef cows (n =

1,358) using the 7 & 7 synch protocol and the 7-day CO-synch + CIDR protocol. The 7 & 7

synch protocol includes a 14-day CIDR inserted with an injection of PGF2α on day 0, followed

by an injection of GnRH on day 7. The CIDR is removed on day 14 along with an additional

injection of PGF2α. There was no difference in estrus expression between the 7 & 7 synch and

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the 7-day CO-synch + CIDR protocols (88 vs 86%, $P = 0.30$). Estrus expression was greatest between 48 and 60 hours post-CIDR removal. However, the 7 & 7 synch protocol had a greater percentage of embryos transferred into recipients that expressed estrus (76% [466/615] vs 65% [418/640], $P < 0.0001$). Pregnancy rates were greater in the 7 & 7 synch group compared to the 7-day CO-synch + CIDR group (40 vs 34%, $P < 0.01$). Thus, it appears that estrus synchronization protocols may impact the likelihood of a recipient female being ready to receive an embryo.

Embryo Transfer

The first successful embryo transfer in cattle was performed surgically in 1949 and non-surgically in the late 1970s (Elsden et al., 1976). Embryo transfer requires the maturity of the embryo to coincide with the number of days post-estrus of the recipient. Successful establishment of pregnancy by embryo transfer can be affected by stage and quality of the embryo, method by which the embryo was produced, and synchrony between embryo and recipient (Wright, 1981).

Wright (1981) evaluated the relationships among embryo factors and recipient factors on pregnancies derived from embryo transfer (n = 2,286). Pregnancy rates from morulae (44%) and advanced morulae (53%) were reduced ($P < 0.05$) compared to early blastocysts (65%), blastocysts (66%) and advanced blastocysts (64%). Pregnancy rates from grade 1 embryos (64%) were greater ($P < 0.05$) than grade 2 embryos (45%) and grade 3 embryos (33%). Pregnancy rates related to synchrony of estrus between donor and recipients were: -36 hours (before donor) (59%), -24 hours (51%), -12 hours (68%), 0 hours (59%), +12 hours (61%), +24 hours (58%), and +36 hours (41%). Difference in pregnancy rates were significant between -12 hours and +36 hours (68 vs 41%, $P < 0.01$).

Spell et al., (2001) evaluated the effects of corpus luteum, concentration of P4 concentration, donor-recipient synchrony, quality of embryo, method of embryo production, and developmental stage of the embryo on pregnancy rates after embryo transfer. In total, 448 cows received an embryo; 122 (27.2%) of which received a fresh embryo, and 326 (72.3%) of which received a frozen embryo. Pregnancy rates were greater ($P < 0.05$) with fresh embryos (83%) compared to frozen embryos (69%). Pregnancy rates were not affected by embryo grade, stage, donor-recipient synchrony, palpated integrity of the CL, diameter of the CL, or concentration of P4. Therefore, based on the results from Spell et al. (2001) suitability of the recipient for embryo transfer should only be determined by observed estrus and a palpable CL regardless of its size or quality.

Follicular Development

Primary oocytes are housed within primordial follicles which remain arrested in the prophase stage of meiosis I until the host reaches sexual maturity (Wandji, 1996). Once sexual maturity is reached, FSH activates a cohort of primordial follicles to develop into primary follicles, whereas LH causes the maturation of follicles (Holesh et al., 2021).

Each follicle contains 2,000 – 20,000 LH and FSH receptors (Yang and Fortune, 2007). Follicular development begins when primary follicles have a layer of 11-20 cuboidal granulosa cells around the oocyte (Yang and Fortune, 2007). Selective primary follicles are recruited by FSH at the beginning of each follicular wave (Rajakoski, 1960). Once these primary follicles are activated and the first meiotic division is completed, the follicle is classified as a secondary follicle. The secondary follicle gains a second layer of granulosa cells and becomes responsive to gonadotropins (Fortune, 2003). The FSH receptor mRNA is localized exclusively in granulosa cells persistently throughout preovulatory follicular development (Hillier et al., 1994).

Oocytes begin to accumulate larger volumes of cytoplasm and develop a zona pellucida (Braw-tal and Yossefi, 1997). Junctional complexes are also developed during this stage between neighboring follicular cells and the oocyte, permitting electronic coupling (Braw-tal and Yossefi, 1997). Junctional complexes between follicular cells are known as gap junctions which aid in nutrient transport, hormonal transfer and electron signaling. Gap junctions remain intact until the preovulatory surge of LH (Broi et al., 2018).

The follicle develops a fluid filled antrum to advance to the tertiary follicle stage (Fortune, 2003). At this step, the LH receptor gene is expressed in the theca cells and the oocyte is considered a secondary oocyte, attaining full cytoplasmic size and the potential to undergo nuclear maturation (Gong et al., 1995).

The "selection" process requires subordinate follicles to undergo atresia. Thus, leaving a single dominant follicle to undergo ovulation. Inhibin is produced by follicles, locally inhibits the growth of surrounding follicles, and suppresses FSH secretion from the anterior pituitary (Gibbons et al., 1997). After "selection", the sources of inhibin are lost, and the selected follicles become E2 -activated (Ireland and Roche, 1982). At this same time, LH receptors also develop in granulosa cells (Xu et al., 1995).

Law et al. (1992) stripped follicular fluid of steroids and found that even without E2, FSH concentrations were suppressed. This suggested that a protein within the follicular fluid known as inhibin was also responsible for the decrease in concentration of FSH. Gibbons et al. (1997) ablated follicles of various sizes and found that follicles 5 mm or larger contributed to the decline in concentration of FSH. The combination of decreased concentration of FSH and increased concentrations of inhibin and E2 leads to atresia of subordinate follicles and selection of the dominant follicle. Although, exogenous FSH can be administered to increase the number

of follicles reaching ovulatory competence. Kimura, (2016) evaluated the effects of super ovulatory protocols including a single subcutaneous injection of FSH in aluminum hydroxide gel and multiple intramuscular injections of FSH dissolved in a saline solution administered twice daily for 4 days. He found that both the single injection and multiple injection super ovulatory protocols produced similar total number of CL (11.0 ± 1.4 and 11.7 ± 1.8 number of CL).

Oocyte maturation is dependent on maturity and functionality of the granulosa and theca cells of the follicle. It is imperative that the dominant follicle attains final maturation as maturation of the follicle is related to maturation of the oocyte (Pohler et al., 2012). Arlotto et al. (1996) evaluated ovulatory follicle size and found that oocytes from follicles greater than 10 mm had increased oocyte diameter compared to oocytes from follicles that were 6 to 10 mm in diameter. Aguila et al. (2020) determined that oocytes acquire developmental competence when the oocyte reaches 120 microns in diameter. Additionally, Arlotto et al. (1996) determined that oocytes from peripheral origin had greater fertilization rates (72 ± 7 vs $62 \pm 8\%$, $P < 0.01$), cleavage rates (69 ± 3 vs $34 \pm 4\%$, $P < 0.01$) and blastocyst rates (25 ± 3 vs $3 \pm 1\%$, $P < 0.01$) compared to oocytes from the cortical region of the ovary.

Overall, larger follicles have increased oocyte competence (Aguila et al., 2020) as well as increased concentration of E2 at estrus and concentration of P4 post ovulation which can lead to increased pregnancy rates (Madureira et al., 2021). FSH promotes follicular growth and development whereas LH promotes follicular maturation (Holesh et al., 2021). Therefore, exogenous FSH increases the size and development of follicles as well as preventing atresia of subordinate follicles. Follicles also secrete inhibin which is primarily responsible for causing apoptosis of subordinate follicles. Once follicles become selected and larger in size, the secretion of E2 increases (Gibbons et al., 1997). Both inhibin and E2 have negative feedback on the

secretion of FSH from the anterior pituitary (Law et al., 1992). This causes a decline in

concentrations of FSH. However, exogenous administration of FSH is used in super stimulatory

protocols to increase the number of follicles reaching ovulation (Kimura, 2016). Therefore, by

increasing endogenous concentration of FSH, follicles that would normally undergo atresia can

be rescued to reach ovulation.

Ovulation

The bovine follicle achieves ovulatory capacity at 10 mm in diameter (Sartori et al.,

2001; Perry et al., 2007), and ovulation usually occurs approximately 35 hours after the onset of

behavioral estrus (Parkinson, 2019). Brewster and Cole (1941) found that the average timing of

ovulation in cows (n = 73) occurred 13.67 ± 0.68 hours after the end of behavioral estrus. Liu et

al. (2018) administered GnRH to induce ovulation and found that the average ovulation time was

30.0 ± 1.0 h post-injection.

Induced ovulation from exogenous GnRH administration is a contributing factor to

decreased pregnancy rates as a sub-mature follicle may ovulate, decreasing oocyte competence,

and post-ovulatory concentration of P4 (Atkins et al., 2010; Perry et al., 2005). Perry et al.

(2005) also found that induced ovulation of follicles that have not reached full ovulatory capacity

negatively affects pregnancy rates and embryonic survival. Vasconcelos et al. (2001) reported

that follicles larger than 15 mm secreted more ($P < 0.05$) P4 from subsequent 7 day old and 14

day old CL compared to follicles smaller than 14 mm (1.61 ± 0.13 vs 1.22 ± 0.15 ng/mL and

3.06 ± 0.15 vs 2.48 ± 0.22 ng/mL, respectively). Additionally, smaller follicles (<11.5 mm)

subsequently form a smaller CL (Vasconcelos et al., 2001; Perry et al., 2007). Follicles induced

to ovulate that were smaller than 11.3 mm in diameter after a GnRH-induced ovulation led to

decreased pregnancy rates (Lamb et al., 2001; Perry et al., 2005; Perry et al., 2007) and increased embryonic mortality rate (Perry et al., 2005).

Mussard et al. (2007) administered GnRH to induce ovulation in cows when ovulatory follicles reached 10 mm. Subsequently, 29 of 29 cows ovulated from the injection of GnRH. Cows administered GnRH (n = 29) to induce ovulation, ovulated sooner than control (n = 24) cows (6.8 ± 0.1 vs 7.7 ± 0.1 days, $P < 0.05$). The control group had increased ovulatory follicle diameter (12.0 ± 0.3 vs 10.7 ± 0.1 mm), CL area (3.6 ± 0.2 vs 3.0 ± 0.2 cm^2), concentration of P4 (6.4 ± 0.3 vs 5.4 ± 0.2 ng/mL) and pregnancy rates (100 vs 76%) compared to the GnRH group ($P < 0.05$; Mussard et al., 2007).

Madureira et al. (2021) administered an estrus synchronization protocol to 1,289 cows along with TAI and found that cows with increased concentrations of E2 at day 0 had a larger ovulatory follicle diameter compared to cows with lesser concentration of E2 (19.5 ± 0.4 vs 18.2 ± 0.3 mm, $P < 0.001$). Cows with greater concentrations of P4 4 days prior to ovulation had larger ovulatory follicles compared to cows with lesser concentration of P4 (14.8 ± 0.2 vs 12.3 ± 0.3 mm, $P < 0.001$). Additionally, cows with greater concentration of P4 at 7 days post-ovulation had larger ovulatory follicles compared to cows with lesser concentration of P4 (13.7 ± 0.3 vs 12.6 ± 0.3 mm2, $P < 0.001$). Pregnancy rates were greater ($P < 0.01$) in cows with increased concentrations of P4 4 days prior to ovulation (32.8 ± 4.4 vs 22.4 ± 4.5%), decreased concentrations of P4 at day 0 (35.2 ± 3.4 vs 19.6 ± 4.6%), and increased concentrations of P4 7 days post-ovulation (39.1 ± 2.9 vs 24.7 ± 2.6%).

Overall, these results indicate that the diameter of the ovulatory follicle is positively correlated with concentrations of P4 during follicular development, concentrations of E2 on day 0, subsequent CL area, post-ovulatory concentrations of P4 and pregnancy rates. Furthermore,

pregnancy rates are increased when concentrations of P4 during follicular development and post-ovulatory concentrations of P4 are increased. Conversely, increased concentrations of P4 on day 0 negatively impacts pregnancy rates.

Corpus Luteum

Vigor of the CL is dependent on the vascularity developed by angiogenic factors during ovulation and CL formation (Fraser, 2006). Therefore, luteal function may be related to the competence of the follicular cells donated by the ovulatory follicle (McNatty et al., 1979). Insufficient luteal function can lead to suboptimal P4 secretion which is a main contributor to pregnancy failure (Shah and Nagarajan, 2013).

During CL regression the correlation between CL size and concentration of P4 decreases because the secretion of P4 decreases more rapidly than physical degradation of the CL (Kastelic et al., 1990; Assey et al., 1993). Circulating concentrations of P4 have a greater correlation with perfusion of blood of the CL compared to the size of the CL (Herzog et al., 2010; Balaro et al., 2017; Rocha et al., 2017).

Pugliesi et al. (2016) studied the impact of the size and perfusion of blood of the CL at embryo transfer on pregnancy rates in recipient cows (n = 329). Recipients were retrospectively divided into two groups according to size of the CL (small [< 3 cm^2] or large [≥ 3 cm^2]) and subdivided into 3 subgroups according to perfusion of blood of the CL (low [$\leq 40\%$], medium [40-50%], or high [$\geq 50\%$]). It was determined that perfusion of blood, not size of CL, affected pregnancy rates (low, 45.1% [37/82]; medium, 55.9% [57/102]; high, 62.3% [38/61]; $P < 0.05$). Overall, perfusion of blood of the CL was increased in pregnant cows compared to non-pregnant cows (48.56 $\pm$ 0.64 vs 46.36 $\pm$ 0.77%, $P < 0.05$).

Velho et al. (2021) synchronized estrus in beef heifers (n = 89) and performed TAI on day 0. On day 5, CL were evaluated for blood perfusion of the CL, diameter of the CL, and concentrations of P4. Luteal blood perfusion was assigned a score of 1 to 3 (1 = minimal; 2 = intermediate; 3 = high blood perfusion). They found that heifers with LBP score of 2 and 3 had increased ($P < 0.01$) CL diameter compared to heifers with LBP score of 1 (17.09 ± 0.37 and 16.41 ± 0.64 vs 13.83 ± 0.47 mm, respectively).

Overall, luteal blood perfusion is a better indicator of the circulatory concentration of P4 compared to luteal size and has been found to increase pregnancy rates. Blood perfusion is required to carry nutrients to the luteal cells for progesterone synthesis and to carry P4 from the luteal cells to the target organ. Therefore, when transferring embryos, recipients with increased luteal blood perfusion may be selected for an increased likelihood to establish pregnancy.

Progesterone

Progesterone stimulates secretions from the endometrial glands of the uterus, contributing secretory products to the uterine environment that support the development of the conceptus (Geisert et al., 1992, Spencer and Bazer, 2004, Spencer, 2014). Progesterone also inhibits myometrial contractions of the uterus creating "uterine quiescence" (Soloff et al., 2011), which is essential for conceptus attachment (Soloff et al., 2011). Another vital role of P4 is to create a "uterine block" in which P4 inhibits the synthesis of oxytocin receptors in the uterine endothelial cells, thus inhibiting the secretion of PGF2α from the uterine endometrium (McCracken et al., 1984).

Interferon Tau (IFN-τ) is a glycoprotein produced by trophoblast cells from the conceptus and essential in blocking the formation of E2 and oxytocin (OT) receptors in the

uterus (Farrin et al., 1990). This is essential to inhibit PGF2α secretion and to inhibit luteolysis. Interferon Tau is detectable as early as day 12 post fertilization in the uterus; however, IFN-τ reaches peak concentrations between days 14-16 which is around the time of conceptus elongation, and just before luteolysis occurs (Mann et al., 1998). Mann and Lamming, (2001) collected embryos 16 days post-insemination and found that cows with a delayed increase of P4 post-ovulation (1 ng/mL^{-1} on day 5.6 ± 0.4) had undetectable concentrations of IFN-τ at day 16 and cows without this delay in P4 had detectable concentrations of IFN-τ (1 ng/mL^{-1} on day 4.1 ± 0.1, $P < 0.01$). Mann and Lamming (2001) also found that cows with a delayed post-ovulation increase in concentration of P4 also had lesser peak concentration of P4 in the luteal phase (6.1 ± 0.4 vs 7.8 ± 0.8 ng/mL^{-1}, $P < 0.05$) compared to cows with an advanced increase in concentration of P4. Additionally, Starbuck et al. (2001) also found that non-pregnant cows experienced a delayed (1.7 day) post-ovulatory rise in concentrations of P4 compared to the pregnant group ($P < 0.001$).

Therefore, an early rise in concentration of P4 is needed in early stages of embryogenesis to promote growth and development of the conceptus as well as increasing the secretion of IFN-τ from the conceptus at day 16. An early rise in concentration of P4 also leads to a greater peak concentration of P4 during the luteal phase which is important for stimulating uterine secretions, and inhibiting E2 and OT receptor formation, therefore inhibiting the secretion of PGF2α and preventing luteolysis

Supplementation with Progesterone

Various methods have been employed to provide additional P4 including intramuscular injection, CIDR, and GnRH- and human chorionic gonadotropin (hCG)-induced ovulation on

day 5 and 6 post-ovulation. Mann and Lamming (1999) conducted a meta-analysis of 17 studies evaluating the effects of supplementation with P4 via intramuscular injection at different time periods and concluded that supplementation with P4 increased pregnancy rates overall by 5%.

Garret et al. (1988) supplemented exogenous P4 via intramuscular injection for 4 days (beginning 36 hours after ovulation, in 24-hour intervals) and found an increase in peripheral plasma concentration of P4 between day 2 to 5 of pregnancy in treated compared to control cows (3.40 ± 0.09 vs 1.22 ± 0.09 ng/mL, $P < 0.001$). Mann et al. (2006) found that early (days 5 to 9) and late (days 12-16) supplementation with P4 via intravaginal CIDR-B device increased plasma concentration of P4 compared to control (6.1 ± 0.5 vs 3.4 ± 0.5 ng/mL and 10.7 ± 0.3 vs 7.4 ± 0.3, respectively). Furthermore, early supplementation with P4 increased trophoblast length ($P < 0.01$) and uterine concentration of IFN-τ ($P < 0.05$) compared to control cows (Mann et al., 2006). Starbuck et al. (2001) found that cows supplemented with P4 via CIDR had improved pregnancy rates ($P < 0.001$) when cows had low concentrations of P4 (1 to 2 ng/mL) on day 5 post-insemination but did not improve pregnancy rates when cows had increased concentration of P4 on day 5 (≥ 3 ng/mL).

García-Guerra et al. (2020) administered GnRH to heifers (n = 360) on day 5 prior to embryo transfer to induce the formation of an accessory CL and evaluate the effects on concentration of P4. They found that 83.9% (302/360) of the GnRH-treated heifers had an accessory CL on day 7. As expected, concentration of P4 were greater in the GnRH-treated heifers compared to control heifers on day 12 (7.2 ± 0.1 vs 6.0 ± 0.1 ng/mL, $P < 0.001$), and day 21 (9.5 ± 0.2 vs 7.4 ± 0.2 ng/mL, $P < 0.001$). Volume of the CL was also greater in GnRH-treated heifers compared to control heifers on day 12 ($7,995 \pm 151$ vs $5,639 \pm 120$ mm^3, $P < 0.001$), and day 33 ($6,805 \pm 116$ vs $5,143 \pm 103$, $P < 0.001$). Although, pregnancy rates on day

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33 did not differ ($P = 0.90$) between GnRH-treated heifers (37.0%, 294/794) and control heifers (37.5%, 288/768).

The results by Garcia et al. (2020) indicate that an accessory CL induced by GnRH on day 5 had no effect on pregnancy rates. However, as concluded by Mann and Lamming (2001), an early increase in concentrations of P4 is required to promote conceptus growth and development. Therefore, a GnRH-induced accessory CL on day 5 may not provide increased concentrations of P4 in a timely manner during early embryogenesis to promote the growth and development of the conceptus needed for the establishment of pregnancy. However, Nishigai et al. (2002) administered hCG to 40 cows on day 6 post-AI and determined that cows that received hCG had increased pregnancy rates compared to the control group (67.5 vs 45.0%, $P < 0.05$).

Mussard et al. (2007) performed dominant follicle ablation on all follicles greater than 4 mm. Cows were then administered two injections of PGF2α to synchronize estrus. Dominant follicles were evaluated using ultrasonography. When the largest follicle reached 10 mm GnRH was administered to induce ovulation in the GnRH group (n = 29). In the spontaneous group follicles were evaluated until estrus was observed. Cows in the GnRH group were artificially inseminated 12 hours after the administration of GnRH and cows in the spontaneous group were artificially inseminated 12 hours after the onset of standing estrus. Interestingly, double ovulations occurred in 47% of the cows. Cows with double ovulations had increased ($P < 0.05$) concentration of P4 and increased ($P < 0.05$) cross sectional area of the CL. Additionally, pregnancy rates were greater ($P < 0.05$) in cows ovulating 2 follicles compared to cows ovulating a single follicle (96 vs 79%).

Thus, supplementation with P4 has been found to increase concentration of P4, trophoblast length, secretion of IFN-τ, and pregnancy rates when cows had low concentrations of

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P4 during the early luteal phase. However, the timing of supplementation of P4 can affect the impact it has on conceptus development and pregnancy rates. When looking at the study by Mussard et al. (2007), when double ovulation occurred naturally, cows had increased concentration of P4 and pregnancy rates. Therefore, a rise in concentration of P4 coinciding with the initial development of the original CL may be required to see positive effects on pregnancy rates.

Luteolysis

Bazer et al. (2012) found that sub-luteolytic pulses of PGF2α are secreted from the uterine endometrium beginning on day 14-15 in both cyclic and pregnant cows. However, a critical number of pulses of PGF2α are required within a given period to induce complete luteolysis (McCracken et al., 1999). In fact, it has been shown that during luteolysis PGF2α is released from the uterus in a series of episodic surges lasting about one hour with approximately 5 pulses in each 24-hour period (Thorburn et al., 1973). Schramm et al. (1984) administered infusions of PGF2α and found that 5, 1-hour long pulses in a 24-hour period caused complete luteolysis, whereas only one single pulse of PGF2α given once a day for 4 consecutive days only caused a temporary fall in P4 followed by recovery of concentrations of P4. These results suggest that frequency of pulses rather than amplitude is required for physiological regression of the CL.

PGF2α is transported from the uterus to the ipsilateral ovary through a vascular countercurrent exchange system (McCracken et al., 1999). In this system, PGF2α secreted by the endometrium enters the uterine vein and the uterine lymph system (Heap et al., 1985). The transfer of PGF2α from high concentrations in the uterine vein to the ovarian artery is facilitated by prostaglandin transport protein (Heap et al., 1985). This allows PGF2α to exert its luteolytic

effect directly on luteal cells of the CL without dilution in systemic circulation (Heap et al., 1985). PGF2α binds to specific receptors on the plasma membrane of luteal cells. The PGF2α receptor complex opens a Ca^{++} channel to allow an influx of high intracellular Ca^{++} to induce apoptotic effects in the cell (Wright et al., 2014). The PGF2α receptor complex also activates a protein kinase-C which inhibits P4 synthesis (McCracken et al., 1999).

Large luteal cells synthesize and secrete oxytocin (OT) from secretory granules, analogous to those of the posterior pituitary (Fields et al., 1992). Schams et al. (1985) hysterectomized cows and found that OT pulses were not observed during expected luteolysis indicating that PGF2α uterine secretion was needed to initiate OT release. Additionally, heifers treated with an OT receptor antagonist, affected neither luteolysis, nor estrous cycle duration (Kotwica et al., 1997). Therefore, OT is not required to initiate pulsatile secretion of PGF2α but does play a supportive and modulatory role in a positive feedback loop of induced uterine PGF2α secretion (Kotwica et al., 1998).

Other factors that affect the synthesis and secretion of PGF2α include tumor necrosis factor-α (TNF-α), IFN-τ and membrane bound enzymes (Mathew et al., 2019). Tumor necrosis factor-α stimulates PGF2α secretion, suggesting that TNF-α is a factor in the initiation of luteolysis (Mathew et al., 2019). Mathew et al., (2019) took endometrial slices at different stages of the estrous cycle and found that OT initiated PGF2α secretion during the follicular stage (days 19-21) and at estrus ($P < 0.001$) but not during the luteal phase. Alternatively, Mathew et al. (2019) tested TNF-α at the same stages and found that it stimulated PGF2α secretion at all stages of the estrous cycle including the luteal phase ($P < 0.001$). Additionally, when endometrial tissues were simultaneously exposed to TNF-α and OT, the stimulatory effect on PGF2α output was increased compared to that of TNF-α and OT alone ($P < 0.05$) (Mathew et al., 2019).

Mathew et al. (2019) also found that PGF2α was increased during the follicular phase and had the highest concentrations during estrus.

Interferon tau produced by trophoblast cells of the conceptus suppress the action of TNF-α on PGF2α synthesis by the endometrium, suggesting that IFN-τ plays a luteo-protective role by inhibiting TNF-α induced PGF2α production in early pregnancy (Okuda et al., 2002). Interferon-τ attenuates prostaglandin secretion in epithelial cells, which are the primary source of PGF2α, by down-regulating COX-2 mRNA (Xiao et al., 1998). However, interferon-τ enhances COX-2 mRNA and prostaglandin synthesis in stromal cells which are the primary source of prostaglandin E_2 (Skarzynski et al., 2000).

Progesterone regulates PGF2α and luteolysis in several ways. P4 regulates the activity of COX-2 and prostaglandin F synthase proteins (Xiao et al., 1998). Progesterone also binds to OT receptors with high affinity, thus inhibiting OT from binding to its own receptor (Bogacki et al., 2000, Zingg et al., 1998). Therefore, the influence of P4 on uterine endometrial responsiveness to OT may occur at different levels, including oxytocin receptor development, G-protein coupling, and second messenger formation (Okuda et al., 2002).

Maternal Recognition of Pregnancy and Pregnancy Maintenance

Maternal recognition of pregnancy of ruminants is a critical sequence of events in which the elongating conceptus must signal its presence to the dam prior to the onset of luteolysis (Swegen, 2021). Embryonic survival is dependent on adequate luteal function, P4 synthesis and responsiveness of the uterus to P4 (Mesen and Young, 2015). Trophectoderm cells develop microvilli that project into the openings of uterine glands (Bazer et al., 2012). The microvilli

immobilize the conceptus and facilitate the uptake of secretions from the uterine glandular epithelium (Bazer et al., 2012).

Initial maternal recognition of pregnancy in cattle occurs on day 16 of pregnancy (Betteridge et al., 1980). The conceptus elongates to occupy the entire ipsilateral horn by day 18 and extends into the horn contralateral by day 24 (Sponchiado et al., 2017). Attachment occurs between days 19-21 and placentation occurs around day 30 (Sponchiado et al., 2017).

One key factor that can influence early pregnancy success are the interactions between maternal environment and the conceptus. The free-floating blastocyst produces anti-luteolytic IFN-τ from the trophectoderm which is the key signaling molecule initiating a cascade of biochemical events that aid in preventing luteolysis (Spencer and Bazer, 2004). Interferon-τ acts on the endometrial cells of the uterus to inhibit the production of OT receptors (Spencer and Bazer, 2004). Interferon-τ also stimulates the secretion of proteins from uterine glands, nourishing and promoting the development of the conceptus (Spencer and Bazer, 2004).

A second mechanism of IFNT is the interferon regulatory factor 2. During the estrous cycle, E2 receptor and OT receptor expression is not detected between days 5 and 11 (Spencer and Bazer, 2004). However, estrogen receptors rapidly increase on day 13, followed by OT receptor on day 14 (Spencer and Bazer, 2004). In uterine epithelial cells, E2 receptor expression increases, and the P4 receptor decreases which allows E2 to induce OT receptor expression, thereby allowing OT to trigger the release of PGF2α pulses (Spencer and Bazer, 2004). The mechanism of interferon regulatory factor 2 allows IFNT binds to IFNTto silence the E2 receptor which prevents E2 induced OT receptor expression and OT induced secretion of PGF2α (Spencer and Bazer, 2004).

Early embryonic loss is the primary cause of pregnancy failure which typically occurs in the first three weeks of gestation (Couto et al., 2019). Most embryonic death occurs during the peri-implantation period and can be attributed to inadequate luteal function, conceptus viability, maternal recognition of pregnancy, implantation, and placentation (Bazer et al., 2012). The endocrine and nutritional status of the dam are critical for successful pregnancy establishment and implantation (Bazer et al. 2012). There is an association between insufficient P4 production from the CL and early embryonic loss (Mann et al., 1999). Mann and Lamming, (2001) demonstrated that the degree of embryo development and IFN-τ secretion is associated with both the timing of postovulatory P4 rise ($P < 0.01$) and peak P4 concentration ($P < 0.05$).

Progesterone acts via the P4 receptor to inhibit the synthesis of OT receptors by uterine epithelial cells known as the period of "P4 block" (McCracken et al., 1984). However, P4 down-regulates its own receptor around day 13 in cyclic and pregnant females (McCracken et al., 1984). This P4 receptor down-regulation allows the expression of E2 receptors which induces the expression of OT receptors and OT induced pulsatile secretion of PGF2α on day 15 and 16 of the estrous cycle (McCracken et al., 1984). Thus, P4 prepares the uterus to produce luteolytic PGF2α if pregnancy is not established. Elli et al. (2001) demonstrated that administration of an anti-prostaglandin agent (ibuprofen lysinate) at embryo transfer increased pregnancy rates (41 of 50 [82%] vs 28 of 50 [56%], $P < 0.05$), thus indicating that suppressing PGF2α secretion favors establishment and maintenance of pregnancy by reducing embryonic mortality.

Most importantly, P4 stimulates the uterus to provide an environment capable of supporting conceptus development and pregnancy establishment (Bazer et al., 2012). Progesterone alters the endometrial transcriptome and modifies conceptus elongation (Carter et

al., 2008). Uterine secretions which make up the composition of uterine luminal fluid (ULF) /

histotroph are secreted by uterine glandular and epithelial cells (Bazer et al, 2012).

The ULF is primarily composed of micro-RNA's, carbohydrates, glucose, amino acids, enzymes,

and signaling molecules (Simintiras and Forde, 2017). Early pregnancy factors produced by the

conceptus include proteins, steroids, and prostaglandins that signal the maternal system to

respond and support pregnancy. These secretions stimulate vasodilation and angiogenesis to

increase uterine blood flow and substrate delivery to the pregnant uterus as well as the transport

of nutrients into the uterine lumen from maternal tissues (Bazer et al., 2012).

Conclusion

In conclusion, P4 produced by the CL is crucial for stimulating increased conceptus

development, secretion of IFN-τ, as well as stimulating changes in uterine secretions and

receptivity. Therefore, increasing the concentration of P4 should enhance the uterine

environment, development of the conceptus, and the ability to establish pregnancy. Progesterone

is also important in preventing the secretion of PGF2α and luteolysis by downregulating the

formation and activity of E2 and OT receptors in the uterine endometrium along with IFN-τ

produced by the conceptus. Supplementation of P4 increases the development of the conceptus

and the secretion of IFN-τ, however, conflicting results have been found when evaluating the

effects of supplementation of P4 on pregnancy rates. The methods of supplementation of P4

include the induction and formation of an accessory CL by the administration of GnRH on day 5,

the use of intravaginal CIDRS, and intramuscular injections of P4 have led to increased

concentration of P4 but did not affect pregnancy rates. Although, cows undergoing double

ovulations and subsequently leading to the formation of 2 CL resulted in increased pregnancy

rates. Therefore, the induction of multiple CL ovulating asynchronously using exogenous FSH to

induce the growth and development of multiple ovulatory follicles may increase concentration of

P4 and be beneficial during the early stages of embryogenesis and CL development which could

lead to increased pregnancy rates in cattle.

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CHAPTER II

INCORPORATING FOLLICLE STIMULATING HORMONE TO STIMULATE MULTIPLE

CORPORA LUTEA FOR EMBRYO TRANSFER IN BEEF CATTLE

Introduction

In 2020, over 1.1 million embryos were transferred in beef and dairy cattle (IETS, 2021).

The average pregnancy rate from conventional frozen embryo transfer in beef cattle is 58.4%

(Hasler, 2001). Although advances have been made in embryo collection techniques, research

has been performed aiming to increase pregnancy rates in embryo transfer, but pregnancy rates

have plateaued over the last several decades. Increased embryo transfer pregnancy rates could

lead to increased economic gain for cattle producers. Embryo transfer has increased cost

compared to other breeding techniques, but increased profits can be expected considering the

ability to multiply desirable genetics from elite dams and sires. The cost of embryo production

along with estrus synchronization cost and technician fees can add up to an average of $435 per

transfer (Facioli et al., 2020; López-Gatius, 2021). The estimated cost of all the embryos

transferred in 2020 was $478,500,000 (Facioli et al., 2020; IETS, 2021; López-Gatius, 2021).

Failure to establish pregnancy can be economically detrimental to embryo transfer programs.

Based on the total cost of embryos transferred in 2020, a 10% increase in pregnancy rates would

return cattle producers 35 million dollars in pregnancies. For this reason, maximizing embryo

transfer pregnancy rates is economically and genetically crucial in seedstock cattle operations.

To establish pregnancy, several physiological changes need to occur. Follicle stimulating

hormone (FSH) and luteinizing hormone (LH) are critical for growth and development of

follicles. Traditionally, concentration of FSH decreases leading up to ovulation, thus permitting subordinate follicles to undergo atresia and result in the ovulation of a single dominant follicle. However, super-ovulatory protocols include the use of exogenous FSH to induce multiple follicles to reach ovulatory competence, thus resulting in the formation of multiple corpus luteum (CL).

The CL is the primary structure for maintaining pregnancy by secreting progesterone (P4) which is crucial for providing a uterine environment capable of supporting embryonic development by stimulating embryotropic uterine secretions (Geisert et al., 1992, Spencer and Bazer, 2004, Spencer, 2014). Progesterone also plays a crucial role in preventing luteolysis by inhibiting oxytocin receptors and the secretion of PGF2α (McCracken et al., 1984). Concentration of P4 can't yet be evaluated chute side and therefore negates the ability to select recipients based on such characteristics. However, other techniques can be used to identify recipients that may be better suited to establish pregnancy such as size and blood perfusion of the CL which are positively correlated with concentration of P4 (Pugliesi et al., 2016). It is clear a relationship exists among concentration of P4, size and blood perfusion of the CL, and establishment of pregnancy.

The objective of this study was to examine the effects of exogenous FSH used in conjunction with an estrus synchronization protocol on CL quantity, area and blood perfusion of the largest CL, as well as circulating concentrations of P4. We hypothesize that exogenous FSH will increase the number of CL, area and blood perfusion of the largest CL as well as circulating concentration of P4, therefore resulting in increased pregnancy rates among embryo transfer in recipient beef cows.

Materials and methods

All procedures in this study were approved by the Institutional Animal Care and Use Committee of Mississippi State University and followed the FASS Guide for the Care and Use of Agricultural Animals in Research and Teaching (IACUC # 20-340).

Animals and treatment

Initially, 80 cows received ultrasound exams on day -19 and -9 to determine cyclicity based on the presence of a CL in an ovary. The first 40 cows identified to be cyclic were included in this project (n = 40; 2 to 10 years, mean of 5 years; between 43 to 122 days postpartum, mean of 72 days). Of these 40 cows, 2 cows were primiparous and 2 cows were nonlactating (stratified between treatment groups). The remaining cows were multiparous and lactating. This research was performed at the H. H. Leveck Animal Research Center – Beef Unit (Mississippi State, MS). Cows were randomly assigned to 1 of 2 treatment groups: control (n = 20; CON) or FSH (n = 20; FSH). On day -9, all cows received GnRH (2 mL IM; Factrel, Zoetis, Kalamazoo, MI, USA) and a vaginal insert containing 1.38 g of P4 (Eazi-Breed CIDR, Zoetis). Cattle assigned to the FSH treatment began receiving their treatment on day -6 at 7 PM and ending on day -2 at 7 AM during CIDR removal. Treatment in the FSH group included 4 injections, 12 hours apart, of 1.0 mL FSH (IM; Folltropin, Vetoquinol, Fort Worth, TX, USA) followed by 4 injections, 12 hours apart of 0.5 mL FSH. Cows in the CON group were not administered injections during the time of FSH administration. On day -2 at 7 AM, the CIDR was removed from all cows and an injection of PGF2α (5 mL IM; Lutalyse, Zoetis) was administered along with the application of an Estrotect patch (Estrotect Breeding Indicator, Estrotect Rockway Inc, Spring Valley, WI, USA) to aid in detection of estrus. Cattle were

observed for estrus on days -1, 0, and 1. Estrus expression was categorized into four 12-hour periods; estrus period 1 (28-40 hours), estrus period 2 (40-52 hours), estrus period 3 (52-64 hours), and estrus period 4 (64-76 hours post CIDR removal). Detection of estrus was evaluated every 6 hours by an experienced evaluator. Cows that did not display estrus (3 FSH, 1 CON) were removed from the study.

Blood samples and analysis

Blood samples were obtained by venipuncture of the coccygeal vein or artery in a 10 mL serum tube (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA). Blood samples were collected on days 7, 14, 21, and 28, and were immediately placed on ice until centrifugation. Samples were centrifuged for 20 min at $4,000 \times g$ at 4°C, serum was separated and then frozen at -20°C for later analysis. Serum samples were analyzed for concentration of P4 using radioimmunoassay kits for P4 (Progesterone Coated Tube Kit, MP Biomedicals, Solon, OH, USA). The intra-assay CV was 7.37%.

Ultrasonography

Transrectal ultrasonography (10.0- to 5.0-MHz linear-array transducer, MicroMaxx, Sonosite Inc., Bothell, WA, USA) was used to measure total number of CL as well as the size and blood perfusion of the largest CL on each ovary on days 7, 14, 21, and 28. Transrectal ultrasonography was also used to determine pregnancy on day 35. To determine blood perfusion, a video clip was recorded of each ovary and three still shots of the cross section of the largest CL was taken. These three images were analyzed using ImageJ (version 1.47; U.S. National Institutes of Health, Bethesda, MD, USA). Data was averaged among the three images. The

percent of area of blood perfusion (ABP) within the largest CL was calculated by counting the pixels of blood perfusion within the total pixels of luteal tissue.

Embryo transfer

Frozen grade 1 conventional embryos were transferred (n = 36) on day 7 according to the guidelines of the International Embryo Transfer Society (IETS, Savoy, IL, USA). Embryos were air thawed for 3 to 5 seconds, then plunged into a thaw bath at 29°C for 20 seconds. Straws were dried using a paper towel and the plug was removed. The straw was then placed into a 21-inch embryo transfer rod. An embryo transfer protective sheath was placed over the rod and a chemise protective plastic covering was placed over the transfer rod for sterility. The chemise was perforated by the transfer gun before advancing into the cervix. Embryos were transferred into the cranial portion of the uterine horn ipsilateral to the largest CL.

Embryos were collected, cryopreserved, and transferred by Bryan Carter at the H. H. Leveck Animal Research Center – Beef Unit. Embryos were produced from 3 donors (2 Angus and 1 Hereford; 1 Angus cow produced 3 matings) from 5 different collections. Each collection was produced by a different sire.

Statistical analysis

Dependent variables included CL quantity, area, and ABP of the largest CL and concentration of P4. Independent variables included treatment, day, and estrus period. The covariates included age and days post-partum. Cow was included in the model as a random variable. Dependent variables were analyzed for normality using the PROC univariate procedure of SAS (SAS software version 9.4; SAS Inst. Inc., Cary, NC, USA) and fitted for normal distribution using the PROC rank procedure of SAS. Initially all variables and 2-way interactions

were included in the model and were removed in a reverse stepwise fashion when *P*-values were greater than 0.10.

The mixed procedure was used for all statistical analyses of dependent variables. The model for all dependent variables included cow (random variable), treatment, day, and estrus period with all possible 2-way interactions.

The model for area of the largest CL included cow (random variable), treatment, day, and treatment by estrus. The model for CL ABP included cow (random variable) and day. The model for concentration of P4 included cow (random variable), treatment, and day. The model for CL total included cow (random variable) and treatment.

Repeated measures analysis was used to analyze the effects over time (d 7, 14, 21, and 28). Covariance structures were tested and those with the lowest AIC and BIC were used for analysis. The first-order autoregressive (AR) covariance structure provided the lowest AIC and BIC fit for CL area, ABP, and concentration of P4. The unstructured (UN) covariance structure provided the lowest AIC and BIC fit statistics for CL total.

Means were separated using the pdiff option of the least square means statement; least squares means and standard errors are reported. Statistical significance was declared at $P \leq 0.05$.

Results and discussion

Expression of estrus

In total, 36 of 40 cows (90%, n = 17 FSH vs. n = 19 CON) were detected in standing estrus (Figure 1). All cows expressing estrus had a CL detected on day 7 prior to embryo transfer. In estrus period 1, 13 cows displayed standing estrus (36%, 6 CON and 7 FSH). In estrus period 2, 11 cows expressed standing estrus (31%, 6 CON and 5 FSH). In estrus period 3,

7 cows displayed standing estrus (19%, 4 CON and 3 FSH). In estrus period 4, 5 cows expressed

standing estrus (14%, 3 CON and 2 FSH).

In this experiment, 86% of cows expressing estrus did so between 28 and 64 hours. The

timing of estrus expression post-PGF2α administration was similar to results found in a study by

Larson et al. (2006) where 81% of cows expressing estrus did so between 36 and 72 hours. The

rates of estrus expression were greater in this study (90% of total recipients expressing estrus,

presenting a CL and received an embryo) compared to Bonacker et al. (2020) where only 56% of

total cows receiving the 7-day CO-Sync+CIDR protocol, and 67% of total cows receiving the

7&7 estrus synchronization protocol had a CL on day 7 and were eligible to receive an embryo.

The increased rates of estrus expression and eligibility for embryo transfer in this study is likely

because only cows determined to be cycling, as having a CL present on day -19 or -9, continued

in the project.

Area of the largest CL (CL area)

Area of the largest CL was affected by day ($P < 0.0001$, Figure 2). The area of the largest

CL on day 7 and 14 was greater than the area of the largest CL on day 21 and 28. Luteolysis

occurs around day 17 in the bovine estrous cycle (Stabenfeldt et al. 1969) in non-pregnant cows

thus causing functional and physical regression of the CL (Okuda and Sakumoto, 2003). Physical

degradation of the CL following luteolysis in non-pregnant cows would explain the overall

decreased area of the largest CL measured on day 21 and 28. This corresponds with results by

Pugliesi et al. (2016) where luteolysis occurred between days 15 and 18 of the estrous cycle

followed by structural luteal regression. During luteolysis, PGF2α is secreted from the uterine

endometrium and binds to receptors on the plasma membrane of luteal cells in the CL thus

allowing an influx of high intracellular Ca^{++} to induce apoptotic effects leading to irreversible degeneration of luteal cells and structural luteal regression (McCracken, 1984).

Area of the largest CL was also affected by a 2-way interaction between treatment and estrus period ($P = 0.0091$, Figure 3). Area of the largest CL was decreased for cows in the FSH group in estrus period 1 compared to all other estrus periods in both treatment groups. In contrast, area of the largest CL was increased for cows in the CON group in estrus period 1 compared to cows in the FSH group in estrus period 1, 2, and 3 as well as cows in the CON group in estrus period 3.

Estrogen is produced by preovulatory follicles and is responsible for inducing expression of estrus (Rodriguez-Martinez et al., 1986). Decreased area of the largest CL in the FSH group in estrus period 1 could be due to inducing multiple follicles to reach ovulation based on the number of CL present on day 7 (3.78 ± 0.9 CL total, FSH and estrus period 1). An increased quantity of ovulatory follicles may produce more E2 and lead to earlier expression of estrus. Ginther et al. (2009) found that cows with multiple ovulatory follicles had a greater concentration of E2, 2 days after follicular deviation. Therefore, multiple ovulatory follicles stimulated by FSH treatment may have produced more E2, inducing an earlier initiation of standing estrus.

Cows in the CON group and in estrus period 1 had larger CL than cows in the FSH group in estrus period 1. These cows most likely had a larger ovulatory follicle. Perry et al. (2007) found that the size of the ovulatory follicle is directly correlated to the size of the subsequent CL. Bridges et al. (2010) found that as the duration of proestrus increased the size of the ovulatory follicle also increased. The results of the current study with increased area of the largest CL in the CON group in estrus period 1 is contradictory to the results found by Bridges et al. (2010).

The initial injection of GnRH at the beginning of the estrus synchronization protocol is intended to induce ovulation and initiate a new follicular wave. However, Sartori et al. (2001) found that ovulatory competence is established when follicles reach 10 mm or greater, and Vasconcelos et al. (1999) found that only 64% of cows ovulated after the first injection of GnRH. This means that 36% of cows did not ovulate and initiate a new follicular wave after the initial injection of GnRH, thus maintaining an advanced follicle at the beginning of estrus synchronization. Colazo et al. (2015) found that cattle that did not respond to the initial administration of GnRH at the beginning of estrus synchronization had larger ovulatory follicles at timed artificial insemination. Therefore, cattle in the CON group in estrus period 1 with a greater area of the largest CL most likely did not respond to the initial injection of GnRH, thus leading to a larger ovulatory follicle at CIDR removal and producing greater concentration of E2, and earlier expression of estrus.

Perry et al. (2007) found that ovulatory follicles less than 10.7 mm and greater than 15.7 mm in diameter resulted in decreased pregnancy rates. Additionally, follicles less than 11.3 mm in diameter (Perry et al., 2007) and follicles greater than 20 mm in diameter (Colazo et al., 2015) result in greater pregnancy loss. Cattle in the FSH group in estrus period 1 had decreased area of the largest CL and cattle in the CON group in estrus period 1 had the greatest area of the largest CL. Therefore, we may expect to see decreased pregnancy rates in estrus period 1 group due to small ovulatory follicles in the FSH group and large ovulatory follicles in the CON group. Pregnancy rates in estrus period 1 were only 31% (4/13), whereas the average pregnancy rate of all cows in the study was 53% (19/36).

Multiple small ovulatory follicles in the FSH group and a single large ovulatory follicle in the CON group may have induced earlier estrus expression most likely due to increased

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concentration of E2. Estrus period 1 resulted in only 31% pregnancy rates which may have been due to the FSH group having multiple small immature ovulatory follicles and the CON group having a single large ovulatory follicle. Therefore, pregnancy rates may be increased by synchronously timing the growth and development of follicles within a herd to reach a medium sized ovulatory follicle at ovulation. The initial administration of GnRH only induces ovulation of mature follicles with ovulatory competence, thus resulting in differences between ovulatory follicle size and maturity after CIDR removal.

Area of blood perfusion of the CL (ABP)

Blood perfusion of the largest CL was not affected by treatment, day, or estrus period ($P > 0.05$). Blood perfusion of the largest CL was similar between FSH and CON groups (16.41 ± 1.9 vs 16.82 ± 1.8 %, $P = 0.8726$). Voelz et al., (2015) found that cattle with 2 CL had greater total luteal blood perfusion compared to cows with only 1 CL ($P < 0.0001$). In this study, treatment with FSH produced a greater total number of CL compared to the CON group (3.48 ± 0.5 vs 0.78 ± 0.5 total CL, $P = 0.0003$), however, we did not see an increase in ABP in the FSH group.

Blood perfusion of the CL is directly correlated to concentration of P4 (Rocha et al., 2017). In the current study there was a slight positive correlation between ABP and concentration of P4 ($r = 0.330$, $P < 0.0001$). Blood perfusion is required to provide nutrients and hormones for the synthesis of P4 as well as transporting P4 from luteal cells to target organs such as the uterine endometrium (Ford, 1982) and furthermore altering the uterine transcriptome. This stimulates embryotropic secretions into the uterine luminal fluid and produces a uterine environment capable of supporting embryo development and pregnancy establishment.

Estrogen has a vasodilation effect and administration of E2 leads to increased uterine blood flow (Roman-Ponce et al., 1978). An increase in the E2 to P4 ratio in systemic circulation also leads to increased blood flow of the entire reproductive tract (Ford, 1982). However, Dysart et al. (2021) found that administration of E2 at estrus increased uterine blood flow but not luteal blood flow.

Size of the CL is directly correlated with size of the ovulatory follicle (Pinaffi et al., 2015). Therefore, larger ovulatory follicles have increased concentrations of E2 and vasodilation effects leading to increased blood flow to the reproductive tract (Roman-Ponce et al., 1978). Treatment with FSH decreased area of the largest CL which had a slight positive correlation with ABP (r = 0.326, $P < 0.0001$). However, in this study treatment with FSH did not affect ABP (16.41 ± 1.9 vs 16.82 ± 1.8, $P = 0.8726$). Area of blood perfusion of the largest CL and pregnancy rates remained similar between treatment groups. Pugliesi et al. (2019) found that increased luteal blood flow led to increased pregnancy rates (low, 46% [62/135]); medium, 54% [93/172]; and high, 58% [80/137], $P < 0.05$).

Concentrations of P4

Concentrations of P4 were different among days of collection ($P = 0.0113$, Figure 4). Concentration of P4 was greater on day 7 compared to day 21 and 28 and greater on day 14 compared to day 21 Functional regression of the CL following luteolysis in non-pregnant cows leads to decreased blood perfusion and concentration of P4 would explain the decreased ABP measured on day 21 and 28.

Concentrations of P4 were increased in cows in the FSH group compared to the CON group ($P = 0.0098$, Figure 5). Treatment with FSH increased the total number of CL which had a positive

correlation with concentration of P4 (r = 0.575, *P* < 0.0001; Table 1). The findings in the current study were similar to Mussard et al. (2007) who found that cattle with multiple CL had a greater concentration of P4 compared to cattle with 1 CL.

Total number of CL

        The total number of CL was greater in cows in the FSH group compared to the CON group (*P* = 0.0003, Figure 6). Follicle stimulating hormone prevents follicular atresia by promoting follicular growth and development and inhibiting apoptosis of follicular cells. Superovulation stimulated by FSH has been used to increase the number of ovulatory follicles in donor protocols (Kelly et al., 1997). Therefore, we expected to find a greater total number of CL in the FSH group compared to the CON group. Luteal cells are responsible for the secretion of P4 (Rocha et al., 2017), thus we expected to see increased concentration of P4 with increased number of CL.

Conclusion

        Area of the largest CL was increased on day 7 and 14 compared to day 21 and 28. However, treatment with FSH decreased the area of the largest CL in cows that exhibited estrus early. In contrast, cattle in the CON group that exhibited early estrus had the greatest area of the largest CL. Area of blood perfusion was not impacted by treatment with FSH, day, or estrus period. Concentration of P4 and CL total were greater in the FSH group compared to the CON group. However, concentrations of P4 were greater on day 7 compared to day 21 and 28, as well as on day 14 compared to day 21. In conclusion, treatment with FSH during estrus synchronization decreased the area of the largest CL and increased concentrations of P4 and total number of CL but had no effect on ABP. Additional research is needed to evaluate the growth of

the dominant follicle and the duration of proestrus. Additionally, further research is necessary to evaluate mechanisms to increase ABP of the CL and its relationship with pregnancy establishment.

Tables and Figures

Table 1   Pearson Correlation Coefficients and Probability for area of the largest corpus luteum (area), area of the corpus luteum with blood perfusion (ABP), concentration of progesterone, and number of corpora lutea (CL total).

Pearson Correlation Coefficients Prob > |r| under H0: Rho=0		
	ABP	CL Total
Area	0.326 $P < 0.0001$	-0.193 $P = 0.0215$
Progesterone	0.330 $P < 0.0001$	0.575 $P < 0.0001$

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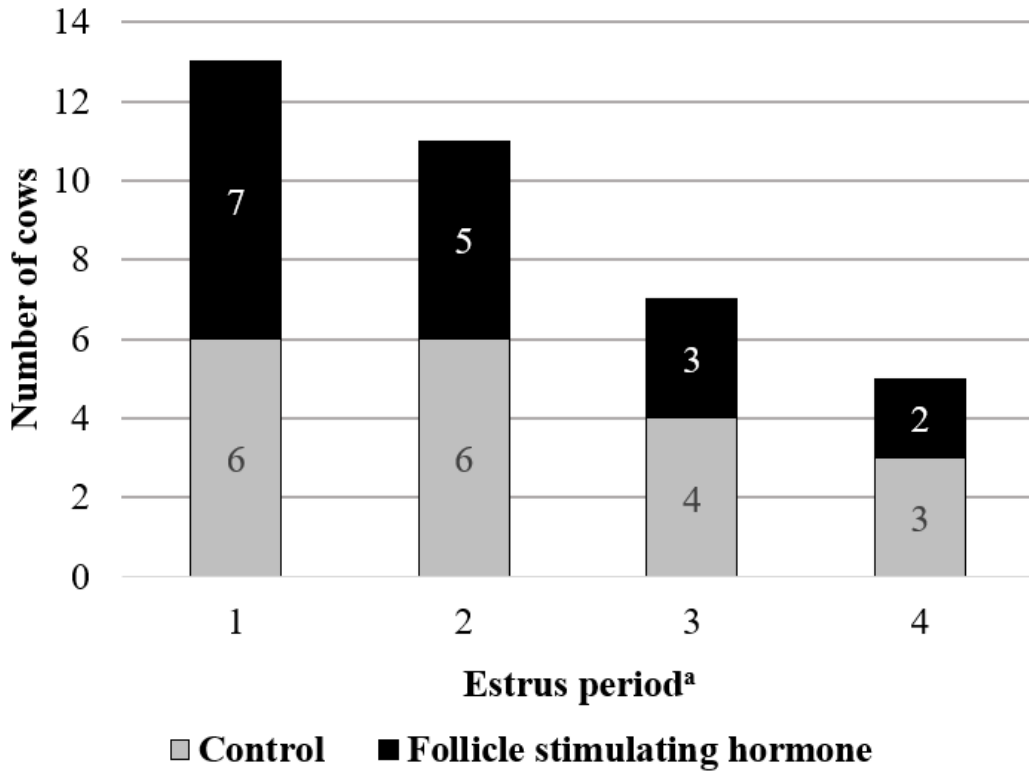
Figure 1   Number of cows observed in estrus in Control or follicle stimulating hormone
(FSH) treatment groups within estrus period[a].

Animals in the FSH group received 8 doses of FSH, 12 hours apart beginning on day -6 at 7 pm
and ending on day -2 at 7 am, d 0 = day of expected estrus.
[a] Timing of first observed estrus in hours post-CIDR removal, categorized into 4 periods (estrus
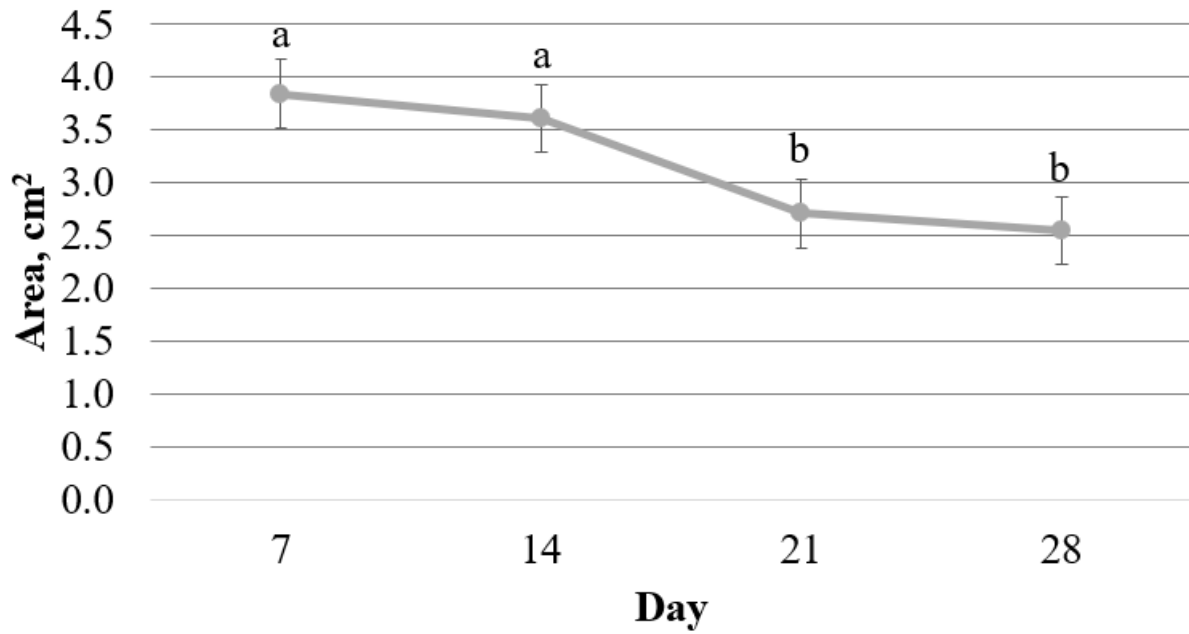period 1: 28-40 hours, estrus period 2: 40-52 hours, estrus period 3: 52-64 hours, and estrus
period 4: 64-76 hours).

Figure 2   LSMeans and standard errors of area of the largest corpus luteum (CL) for all cows on days 7, 14, 21, and 28 ($P < 0.0001$).

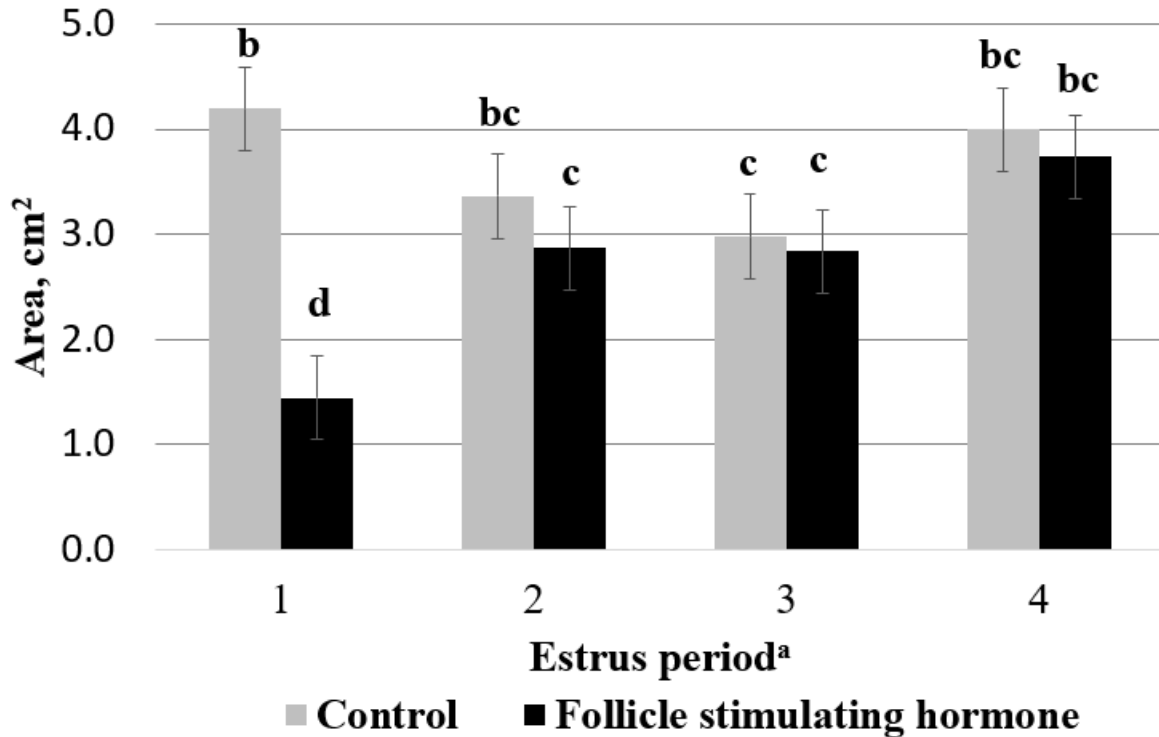[ab] Lack of common superscripts indicate difference of $P < 0.05$.

Figure 3   LSMeans and standard errors of area of the largest CL in control or follicle stimulating hormone (FSH) treatment groups among estrus period[a].

Animals in the FSH group received 8 doses of FSH, 12 hours apart beginning on day -6 at 7 pm and ending on day -2 at 7 am, d 0 = day of expected estrus. Interaction of estrus period and treatment was $P = 0.0044$.

[a]Timing of first observed estrus in hours post-CIDR removal, categorized into 4 periods (estrus period 1: 28-40 hours, estrus period 2: 40-52 hours, estrus period 3: 52-64 hours, and estrus period 4: 64-76 hours).

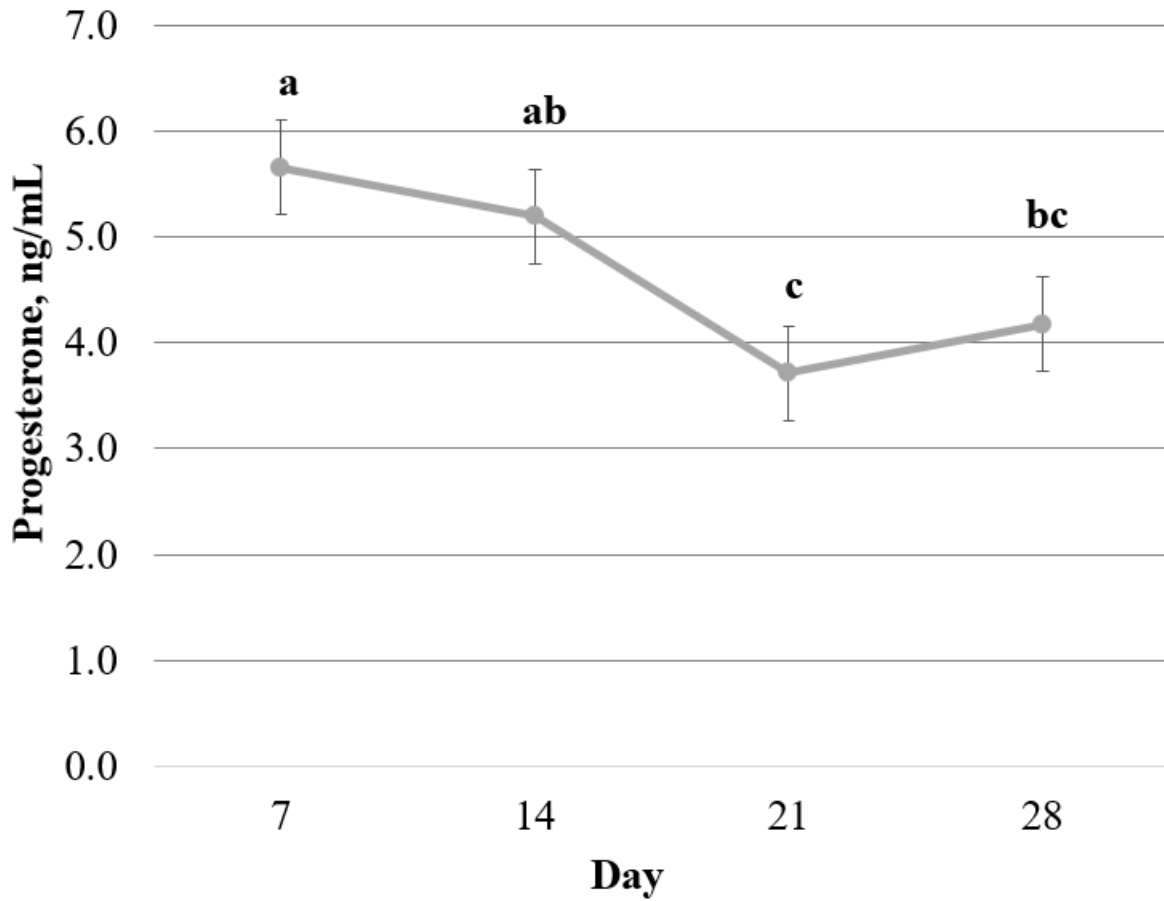[b,c,d] Lack of common superscripts indicate difference of $P < 0.05$.

Figure 4   LSMeans and standard errors of concentrations of progesterone for all cows on days 7, 14, 21, and 28 ($P = 0.0113$).

[a,b,c] Lack of common superscripts indicate a difference of $P < 0.05$).
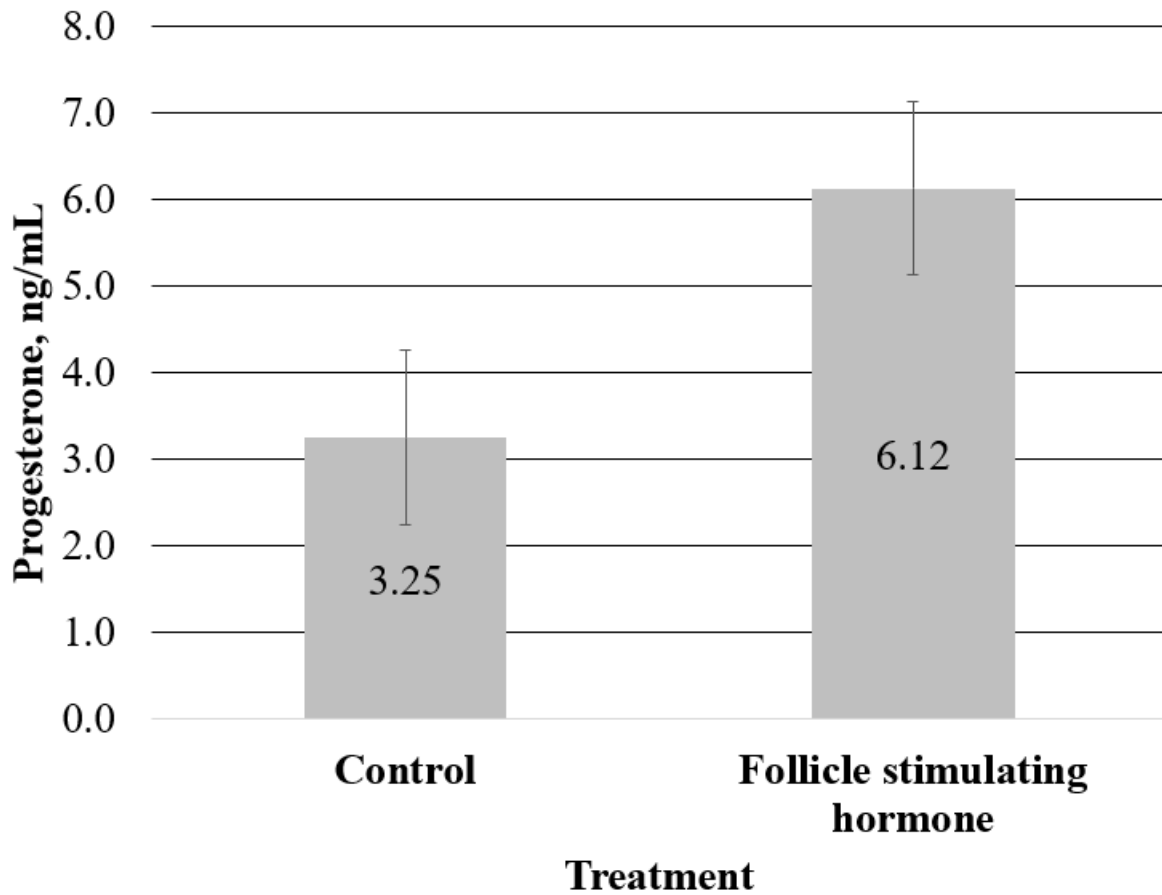
Figure 5   LSMeans and standard errors of concentrations of progesterone between control and follicle stimulating hormone (FSH) treatment groups.

Animals in the FSH group received 8 doses of FSH, 12 hours apart beginning on day -6 at 7 pm and ending on day -2 at 7 am, d 0 = day of expected estrus ($P = 0.0032$).
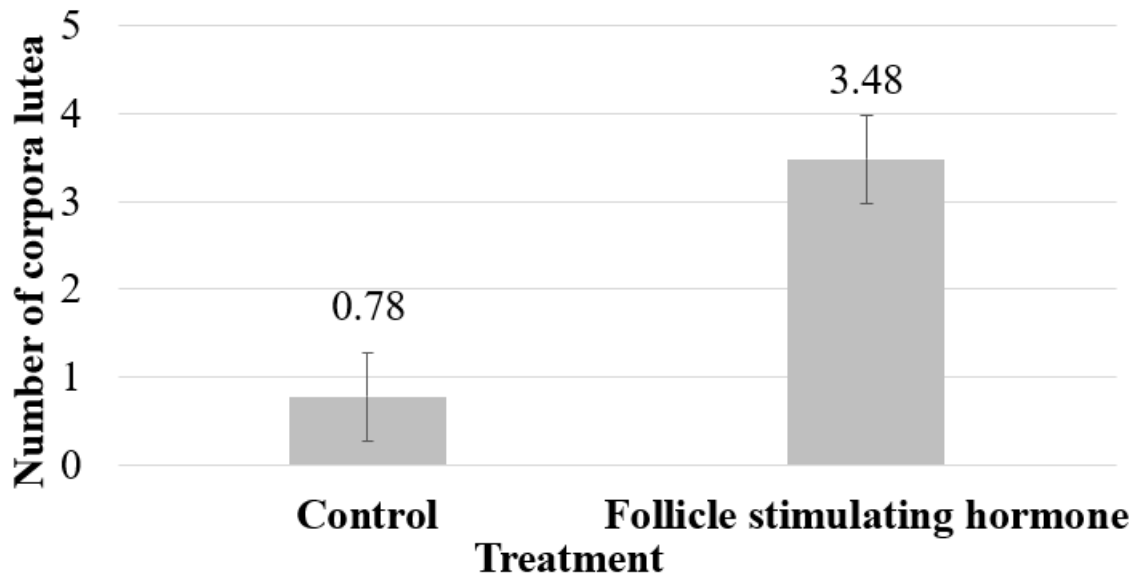
Figure 6   LSMeans and standard errors of number of corpora lutea between control and follicle stimulating hormone (FSH) treatment groups.

Animals in the FSH group received 8 doses of FSH, administered 12 hours apart beginning on day -6 at 7 pm and ending on day -2 at 7 am, d 0 = day of expected estrus ($P = 0.0003$).

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