IMPROVING DAIRY CATTLE FERTILITY:
THE ROLE OF ASPIRIN ON PROSTAGLANDIN SECRETION AND THE USE OF AN ALTERNATE RESYNCHRONIZATION PROTOCOL TO IMPROVE PREGNANCY RATES IN LACTATING DAIRY COWS

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AUTHORIZATION TO SUBMIT THESIS

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ABSTRACT

In cattle, early embryonic loss may occur due to the premature secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) during days 14-16 after fertilization. The objective of the first experiment in this study was to determine aspirin's effects on $PGF_{2\alpha}$ during the luteal phase of the estrous cycle in lactating dairy cows by characterizing blood plasma prostaglandin metabolites (PGFM) and progesterone (P_4) concentrations. Aspirin decreased PGFM for 30 hours after last bolus administration and increased the day to luteolysis. The objective of the second experiment was to determine the effectiveness of the initial gonadotropin-releasing hormone (GnRH) in a 5-day CIDR (controlled internal drug releasing inserts)-Cosynch resynchronization protocol on fertility to second insemination in lactating dairy cows. No differences in pregnancy rate per artificial insemination (PR/AI) were observed between no GnRH (treatment) and GnRH (control) for the second insemination. Indicating that GnRH may not be necessary at the initiation of a CIDR-Cosynch resynchronization protocol.

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DEDICATION

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LIST OF ABBREVIATIONS

150CM	150 Day Corrected Milk
AA	Arachadonic Acid
AI	Artifical Insemination
AMDUCA	Animal Medicinal Drug Use Clarification Act
ARMA	Autoregressive Moving Average
BCS	Body Condition Score
BEND	Bovine Endometrial
BRD	Bovine Respiratory Disease
BW	Body Weight
CI	Conception Interval
CIDR	Controlled Internal Drug Release Insert
CL	Corpus Luteum
COX	Cyclooxygenase
CR	Conception Rate
CV	Coefficients of Variance
DIM	Days in Milk
DIMFB	Days in Milk to First Breeding
DIMSB	Days in Milk to Second Breeding
DO	Days Open
E_2	Estrogen
EIA	Enzyme Immunoassay
ELDU	Extra Label Drug Use

ERα	Estrogen Receptor alpha
FM	Flunixin Meglumine
FSH	Follicle Stimulating Hormone
GLM	General Linear Model
GnRH	Gonadotropin Releasing Hormone
hCG	Human Chorionic Gonadotropin
HD	Heat Detection
IFNτ	Interferon-tau
i.m.	Intramuscular
i.v.	Intravenous
LH	Luteinizing Hormone
MAPK	Mitogen Activated Protein Kinase
NSAID	Non-Steroidal Anti-Inflammatory Drug
OTR	Oxytocin Receptor
OXY	Oxytocin
P_4	Progesterone
P/AI	Pregnancy per Artificial Insemination
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGFM	Prostaglandin Metabolite
PGH_2	Prostaglandin H_2
PGHS	Prostaglandin H Endoperoxide Synthase
PKC	Phosphokinase C

PLA_2	Phospholipase A_2
PR	Pregnancy Rate
RIA	Radioimmunoassay
S/C	Services per Conception
TAI	Timed Artificial Insemination
TBRD	Number of Times Bred
THI	Temperature-Humidity Index
TMR	Total Mixed Ration
VWP	Voluntary Waiting Period

CHAPTER ONE

"The Effects of Aspirin on Prostaglandin Metabolite and Progesterone Concentrations in Lactating Dairy Cows"

ABSTRACT

Approximately 70 to 80% of total embryonic loss in dairy cattle occurs between days 8 and 16 after artificial insemination (AI). Early embryonic loss may be due to the premature secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) during days 14-16 after fertilization. The objectives of this study were to examine the effect of aspirin, a non-steroidal anti-inflammatory drug (NSAID), on $PGF_{2\alpha}$ secretion in lactating dairy cows by characterizing blood plasma prostaglandin metabolites (PGFM) and luteal function by characterizing progesterone (P_4) concentrations during the luteal phase of the estrous cycle. Twenty-three lactating Holstein cows were synchronized to ovulation. Ovulation was confirmed by ultrasonography (days 0, 3, & 7). On day 14, and after detection of corpora lutea, cows were randomly assigned to receive either aspirin (total of 140 g) or no aspirin (control). Subsequently, a blood sample was obtained from each cow. Aspirin was given orally on day 14 (2×) and day 15 (1×), 12 hours apart. On day 15, six hours after the last dose of aspirin, hourly blood samples were taken for 6 consecutive hours for PGFM concentrations. Daily blood samples were also collected (from day 15 to 23) to examine P_4 concentrations and determine day of luteolysis, which was defined as two consecutive days < 1 ng/mL (n = 16). Analysis of repeated measures using the mixed model procedure were conducted using SAS 9.3. The model included treatment, the repeated factor time, and treatment × time interaction. Cow within treatment was considered to be the random effect and body weight (BW) was used as a covariate. Prostaglandin metabolite data was transformed using the

natural log (LN) function in order to stabilize variance and distribution of the data. On day 14, mean P_4 was > 1 ng/mL for all cows and was similar between groups. Before treatment, there were no differences ($P > 0.05$) in mean PGFM concentrations between the groups, (5.1 vs. 5.2 ± 0.2 for aspirin and control, respectively). There was an effect of treatment and treatment × time on mean PGFM ($P < 0.05$). Mean PGFM concentrations were decreased ($P < 0.05$) 30 hours after first aspirin administration and remained low for 5 hours after the last treatment, whereas the mean PGFM concentrations remained unchanged in the control group. Overall, mean PGFM concentrations were significantly different ($P < 0.05$) and were 4.3 vs. 4.9 (SE ± 0.19; $P < 0.05$) for aspirin and control, respectively. Blood P_4 concentrations post-treatment were similar between the two groups (3.6 vs. 3.2 ng/mL; SE ± 0.6), and both groups declined from day 15 to 23 ($P < 0.01$). Although there were no differences in P_4 concentrations, day to luteolysis tended ($P = 0.09$) to be greater for aspirin (n = 8) versus control (n = 8) (20.6 vs. 19.25 ± 0.5 day of estrous cycle). These results indicate that oral administration of aspirin during day 14 to 15 of the estrous cycle suppresses PGFM and may delay luteal tissue regression.

Keywords: dairy cows, NSAID, prostaglandin $F_{2\alpha}$, aspirin

INTRODUCTION

Contemporary dairy cattle have significantly higher milk production than 30 to 40 years ago due to improvements in genetics and nutrition (Sreenan et al., 2001). However, conception rates (CR) following artificial insemination (AI) have steadily declined. This reproductive inefficiency has an enormous impact on the profitability of the dairy industry. First service CR's have fallen from 65% to 40% between 1951 and 1996, which is

approximately 0.3% per year (Butler, 1998; Royal et al., 2000). More recently, it has been reported that from 1977 to 2002 pregnancy rates (PR) have declined from 21.6% to 12% with the lowest PR occurring during the summer months (de Vries and Risco, 2005). Also, the average days to 1st AI have increased by 16 days in the past couple of decades (Washburn et al., 2002). Silvia (1998) reported that in Kentucky, the number of services per conception (S/C) has increased from 1.62 to 2.91 between 1972 and 1996. With more S/C, and lower PR there has been an increase in reproductive management expenses negatively affecting dairy producers profitability.

The modern dairy cow produces a greater volume of milk compared to fifty years ago, and dairy cattle with higher milk production tend to have higher incidences of infertility (Butler, 1998; Lucy, 2001; Cerri et al., 2004; Stevenson et al., 2007; Santos et al., 2009). Although milk production quantity does not directly affect fertility, higher metabolic rates increase the clearance of many hormones including steroid hormones, which are essential to reproduction (Wiltbank et al., 2006). Some recent infertility issues that have been reported include longer days to first ovulation, higher rate of anestrous cows at breeding, longer days of luteal phase (12.9 to 14.8), lower P_4 concentrations, and a greater rate of embryonic mortality (Vasconselos et al., 1997; Pursley et al., 1998; Royal et al., 2000; Stevenson, 2000; Lucy, 2001).

Many physiological events must occur for successful fertilization, implantation, and maintenance of pregnancy. Changes in hormonal profiles can have a significant effect on overall success of pregnancy. For example, early secretion of $PGF_{2\alpha}$ may cause early luteolysis of the CL, which may contribute to the high occurrence of early embryonic death. Approximately, 70 to 80% of total embryonic loss in dairy cattle occurs between days 8 and

16 after AI (Dunne et al., 2000; Sreenan et al., 2001). Early embryonic loss may be due to the premature secretion of $PGF_{2\alpha}$ between days 14 and 16 following fertilization when maternal recognition by the embryo must occur. Collectively, studies suggest that early embryonic losses, which occur near to the time of maternal recognition of pregnancy, may occur because certain embryos are unable to inhibit secretion of $PGF_{2\alpha}$ (Thatcher et al., 2001). Therefore, any strategies, which may inhibit or reduce secretion of $PGF_{2\alpha}$ during early embryonic development (day 13 to 15 after fertilization) may reduce embryonic loss and increase reproductive performance of dairy cattle.

REVIEW OF LITERATURE

ESTROUS CYCLE

In dairy cows, the average length of the estrous cycle is 21 days and ranges from 18 to 24 days. The estrous cycle consists of two phases, follicular and luteal. Based on ovarian status and estrus behavior, the estrous cycle can be divided into four stages, proestrus, estrus, metestrus and diestrus.

Follicular Phase

The follicular phase is approximately 4 days long from day 19 to day 1 (with day 0 considered to be the day of estrus and ovulation) of the estrous cycle. It consists of proestrus (3 days) and estrus (1 day) (Senger, 2012). The main ovarian structure that develops during this phase is a follicle. The granulosa cells of the graffian follicle produce and secrete 17β-estradiol (E_2). The estrus stage is defined as the day of estrual behavior and ovulation (day 0 of cycle) and is denoted by high blood E_2 concentrations. High E_2 increases E_2 receptors as well as P_4 receptors and oxytocin (OXY) receptor expression in the

uterus (Spencer and Bazer, 2004). Elevated E_2, through a positive feedback mechanism, causes a surge of hypothalamic gonadotropin-releasing hormone (GnRH) from the surge center and subsequently the pre-ovulatory surge of luteinizing hormone (LH) from the anterior pituitary (Senger, 2012). Luteinizing hormone causes the ovulation of the follicle and initiates the transformation of granulosa and theca cells into luteal cells.

Luteal Phase

The luteal phase is approximately 18 days long, from day 1 to 19, and consists of the metestrus and diestrus stages of the estrous cycle (Salisbury et al., 1978). The predominant ovarian structure during this phase is the corpus luteum (CL), which produces and secretes P_4 from the small and large luteal cells. During metestrus, the CL begins to develop and by the diestrus stage, the CL is fully functional, and produces maximal P_4. During the late diestrus stage, and in the absence of an embryo, the CL regresses (luteolysis); which is caused by an increase in $PGF_{2\alpha}$ secretion (Senger, 2012). Luteolysis begins when an initial pulsatile release of $PGF_{2\alpha}$ causes a decrease in P_4 secretion and increase in E_2 secretion. Concurrently, luteal tissues begin to secrete OXY and the CL becomes responsive to pulsatile secretion of $PGF_{2\alpha}$. At this time, the luteal cells that make up the CL begins to secrete more OXY which stimulates $PGF_{2\alpha}$ secretion from the uterine endometrium. Prostaglandin $F_{2\alpha}$ is transported via a local venoarterial countercurrent exchange system (Ginther, 1974; Lamming and Mann, 1995). This hormone inhibits P_4 synthesis (inhibiting conversion of cholesterol to P_4) and causes CL regression by causing an influx of calcium and cytokines leading to apoptosis, and tissue necrosis of luteal cells.

GESTATION

Establishment of Pregnancy

In order for pregnancy to be maintained in dairy cattle, P_4 synthesis and secretion must be upheld. To sustain P_4, the uterus must receive a signal from the fetus to inhibit $PGF_{2\alpha}$ secretion and luteolysis. In a non-pregnant cow, OXY mediates pulsatile secretion of $PGF_{2\alpha}$ from the endometrium of the uterus (Walters et al., 1984; McCracken et al., 1999). Prostaglandin F_{2a} is catalyzed by cyclooxygenase and is important for luteolysis, ovarian function, and luteal maintenance during pregnancy. Studies have shown that $PGF_{2\alpha}$ is implicated in ovulation, oocyte maturation, implantation, embryo development, maintenance of pregnancy, and processes of parturition such as cervix dilation and labor (Basu and Kindahl, 1987; Basu et al., 1988). In cyclic sheep, the loss of P_4 receptors is followed by an increase in the epithelial E_2 receptors and OXY receptors (OTR) in the uterus, which ultimately induces the release of $PGF_{2\alpha}$ (Spencer and Bazer, 2004). Prostaglandin $F_{2\alpha}$ is released in a pulsatile manner for the first 2 to 3 days with rapid pulses every 1 to 5 hours before luteolysis and then a consistent secretion during luteolysis (Aiumlamai et al., 1990; Senger, 2012). It has been shown that an injection of $PGF_{2\alpha}$ between day 5 to 8 following AI increases the risk of early embryonic loss (Seals et al., 1998) and also negatively affects fertilization and embryo function (Thatcher et al., 1994).

In a pregnant cow, the embryo (blastocyst) produces a specific protein called interferon tau (IFNτ), which down regulates OTR and ultimately prevents the secretion of $PGF_{2\alpha}$ leading to maintenance of the CL and high concentrations of P_4. High concentrations of P_4 are necessary in order to prime the uterus for embryo implantation, development, and formation of placenta (Spencer and Bazer, 2004). During the diestrus stage, P_4 increases and

binding of P_4 to its receptor blocks expression of estrogen receptor alpha (ER_α) and OTR in the endometrial luminal epithelium and superficial ductal grandular epithelium (Spencer and Bazer, 2004). The expression of both OTR and cyclooxygenase 2 (COX 2) are considered to be rate-limiting steps in the synthesis of $PGF_{2\alpha}$ (Spencer and Bazer, 2004).

Maternal recognition by bovine embryonic IFNτ occurs between days 12 to 25 following fertilization with maximum production occurring between days 15 to 16 post-fertilization (Farin et al., 1990). Given the short time period, maternal recognition must occur in a timely fashion in order to prevent luteolysis. Any unsuccessful interaction between the conceptus and dam, such as delay in fetal development or secretion of IFNτ, or premature secretion of $PGF_{2\alpha}$ prior to embryo signaling, may result in early embryonic loss.

Early Embryo Development

Embryonic development is dependent on both oviductal and uterine environments, and is influenced by oocyte history as well as subsequent CL development (Thatcher et al., 1994). When the conceptus develops into a blastocyst, it travels from the oviduct to the uterus 3 days after conception (Betteridge and Flechon, 1988). Shortly after, hatching of the blastocyst occurs allowing the conceptus to escape the zona pellucida and expose the trophectoderm for attachment to the uterine wall (Roberts et al., 2008). During this time, the embryo elongates from a spherical shape to a tubular and then filamentous shape. By day 14 following fertilization, the hatched blastocyst attaches to the uterine wall and complete attachment occurs around day 19 with caruncular-cotyledons structures of the placenta visible by day 21 (Sreenan et al., 2001).

From day 13 to 16, the embryo significantly increases in size. The length of the embryo increases from 5.25mm to 52mm and the diameter increases from 0.9mm to 1.9mm

(Grealy et al., 1996). This elongation significantly increases the metabolic activity and secretion of IFNτ by the embryo (Farin et al., 1990; Robinson et al., 2006). Interferon-tau is produced by the conceptus during the filamentous stage of development and is released from the mononuclear cells of the trophectoderm (Bazer, 1992; Spencer and Bazer, 2004; Roberts et al., 2008). It has been shown that bovine IFNτ mRNA increases as early as day 12 following fertilization and can last up until day 25 with maximum production between days 15 to 16 (Farin et al., 1990). Upon IFNτ secretion by the embryo, endometrial $PGF_{2\alpha}$ synthesis and secretion is inhibited, thereby maintaining luteal function P_4 concentrations, which help to establish and maintain early pregnancy (Estergreen et al., 1967).

HORMONES

Interferon-tau

In 1992, Roberts recommended the nomenclature for ruminant trophoblastic interferons to be IFNτ (Roberts et al., 2008). Interferon-tau is a trophoblastic protein that is an endometrial intracellular inhibitor of $PGF_{2\alpha}$. Higher concentrations of IFNτ have been reported between day 15 and 16 post fertilization (Northey and French, 1980; Thatcher et al., 1984; Gross et al., 1988; Farin et al., 1990; Spencer and Bazer, 2004). Interferon-tau induces changes within the uterine environment for maintenance of pregnancy by balancing between luteolytic and luteotrophic substances (Thatcher et al., 1984). In bovine endometrial (BEND) cells (*in vitro*), IFNτ increases prostaglandin E_2 (PGE_2), which is a luteotrophic hormone that protects the CL (Asselin et al., 1996). Interferon-tau acts on the endometrium by regulating the gene expression of $PGF_{2\alpha}$ synthesis and secretion (Basu and Kindahl, 1987; Basu et al., 1988; Helmer et al., 1989; Thatcher et al., 1997). It has been shown *in vivo*, that both ovine and bovine recombinant IFNτ can extend the CL lifespan and prevent

oxytocin-induced $PGF_{2\alpha}$ secretion in cyclic cows (Meyer et al., 1995). Therefore, any mechanism that prevents $PGF_{2\alpha}$ secretion by the uterus may prevent early regression of the CL and early embryonic loss.

Prostaglandins

Prostaglandins are synthesized by the oxidation of arachadonic acid (AA) by cyclooxygenase (COX) 1 and 2 enzymes. These enzymes are important for stimulation of inflammatory cells, uterine contractions and regression of the CL (Elli et al., 2001). Cyclooxygenase-1 mediates the formation of prostaglandin including gastrointestinal cells, platelets, endothelial cells and renal cells, which can be affected exogenously (Agrawal and Gupta, 2010). Cyclooxygenase-2 is inducible and catalyzes the formation of intermittently needed prostaglandins such as $PGF_{2\alpha}$ (Agrawal and Gupta, 2010). Substances derived from COX enzymes are involved in many reproductive events such as endometrial vascularity, blastocyst hatching, embryo implantation and decidualization (Elli et al., 2001).

Prostaglandin $F_{2\alpha}$ is an oxygenated polyunsaturated 20-carbon fatty acid with a cyclopentane ring and is derived from the precursor AA (Basu, 2007). Prostaglandin $F_{2\alpha}$ is formed by the reduction of prostaglandin H_2 (PGH_2) by prostaglandin endoperoxide H synthase or reductase (PGHS) (Burns et al., 1997). In the uterus, the events involving $PGF_{2\alpha}$ synthesis and secretion are mediated by luteal OXY binding to the OXY receptor (OTR) on the plasma membrane of endometrial cells. Oxytocin plays a critical role in $PGF_{2\alpha}$ synthesis and secretion. Upon binding of OXY to the OTR in the endometrium of the uterus, phosphokinase C (PKC) is activated and increases phospholipase A_2 (PLA_2) activity and intracellular calcium (Burns et al., 1997). When bovine endometrial (BEND) cells *in vitro* were stimulated with phorbol ester, a compound mimicking intracellular events for $PGF_{2\alpha}$

synthesis, $PGF_{2\alpha}$ synthesis was increased (Thatcher et al., 2001). In this study, the increase in $PGF_{2\alpha}$ synthesis was demonstrated to be controlled by the mitogen activated protein kinase (MAPK) pathway, which also activates PKC. As previously mentioned, PKC is also responsible for increasing intracellular calcium and COX-2 gene expression (Thatcher et al., 2001).

Estradiol 17β also plays a pivotal role in $PGF_{2\alpha}$ secretion. Estradiol 17β up regulates ER_{α} intracellularly, which subsequently stimulates OTR gene expression at the plasma membrane of the uterine endometrium (Spencer and Bazer, 2004). When OTR are up regulated, more binding sites become available for OXY binding, resulting in more gene expression for COX enzyme for $PGF_{2\alpha}$ production. Inhibition of $PGF_{2\alpha}$ production can occur by high concentrations of P_4 as well as the presence of IFNτ. Both hormones down regulate ER_{α} expression and therefore, decrease the number of OTR and the amount of COX available for synthesis of $PGF_{2\alpha}$. However, ER_{α} is also important for increasing the number of P_4 receptors, and hence, a down regulation in ER_{α} expression decreases the number of P_4 receptors in the uterine endometrium.

The rate of $PGF_{2\alpha}$ secretion is dependent upon AA availability and the activity of prostaglandin H synthase (PGHS) (Thatcher et al., 1997). Phospholipase A_2 (PLA_2) liberates AA from the plasma membrane of endometrial cells and a decrease in PLA_2 decreases the availability of AA, therefore decreasing the synthesis and secretion of $PGF_{2\alpha}$ (Meyer et al., 1996; Thatcher et al., 2001). Given that PLA_2 is important for AA availability, it is considered to be the rate-limiting enzyme for $PGF_{2\alpha}$ synthesis.

The stable metabolite of $PGF_{2\alpha}$ in circulation is plasma prostaglandin metabolite (PGFM) (Zollers et al., 1993). A study in ovine species demonstrated that jugular PGFM

concentrations were correlated to uterine-ovarian $PGF_{2\alpha}$, and therefore could be used to assess $PGF_{2\alpha}$ concentrations indirectly (Mitchell et al., 1976). Similarly, research in bovine species has shown that peripheral PGFM is correlated to uterine-ovarian $PGF_{2\alpha}$ and therefore can also be used as an index for uterine $PGF_{2\alpha}$ production (Kindahl et al., 1976 & 1981; Thatcher et al., 1984).

EARLY EMBRYONIC LOSS

Embryonic loss is classified as early or late; early embryonic loss occurs shortly after conception, whereas late embryonic loss is described as at or beyond the filamentous stage of embryo development (Lucy, 2001). Furthermore, the phrase "early embryonic loss" has been defined as loss of pregnancy prior to day 24 after fertilization (Humbolt, 2001). In cattle, the majority of embryonic loss occurs during early embryonic development with approximately 70-80% of total embryonic loss occurring between days 8 and 16 (Dunne et al., 2000; Sreenan et al., 2001; Silke et al., 2002). Furthermore, using ultrasonography, late embryonic loss has been shown to account for 20% of total embryonic loss and occurs between day 28 and 60 following fertilization (Vasconcelos et al., 1997; Pursley et al., 1998). In fact, it has also been shown that for moderate milk producing dairy cows, the fertilization rate is approximately 90% with an average of 55% calving, indicating an embryonic mortality rate of 40% between fertilization and parturition (Thatcher et al., 1994; Sreenan et al., 2001).

Many physiological events must occur for proper fertilization and maintenance of pregnancy. Some studies have shown that early embryonic loss is associated with shorter cycles; mainly due to a shorter lifespan of the CL which causes an altered uterine environment increasing the risk of early embryonic death (Thatcher et al., 1994). High P_4

and low E_2 during metestrus prepares the uterus for early embryonic development, implantation, placentation, and contributes to the overall success of fetal and placental development until parturition (Spencer and Bazer, 2004). Early embryonic loss accounts for approximately 20.5% to 43.6% of increased number of AI's before conception occurs (Humbolt, 2001). Some studies have suggested that early embryonic loss may be due to lower oocyte quality and/or the age of the follicle prior to ovulation and fertilization (Humbolt, 2001). However, a delay of embryo signaling, subnormal P_4 concentrations, or premature secretion of $PGF_{2\alpha}$ may also play a critical role in early embryonic loss.

Low post-ovulatory P_4 concentrations have been associated with embryonic loss (Sreenan et al., 2001). Prostaglandin $F_{2\alpha}$ concentrations are greater in cattle with low P_4 rather than high P_4 (Mann and Lamming, 1995). Given that high producing dairy cattle have elevated metabolic rates and a rapid clearance of hormones (Wiltbank et al., 2006), the concentration of P_4 may be suboptimal and therefore may cause an elevation in $PGF_{2\alpha}$ concentrations. If premature secretion of $PGF_{2\alpha}$ occurs during early embryo development, studies have indicated a negative association with embryo quality as well as the overall success and maintenance of pregnancy (Schrick et al., 2001).

Low levels of P_4 prior to ovulation, and premature secretion of E_2 by the follicle causes an upregulation of OTR in the endometrium resulting in premature $PGF_{2\alpha}$ synthesis and secretion (Inskeep, 2004). In the event of low P_4 and high E_2, there is an increase in OXY followed by an increase in PGFM concentration which indirectly indicates an increase in $PGF_{2\alpha}$ (Kindahl et al., 1976 & 1981; Thatcher et al., 1984). These abnormal hormonal patterns and abnormal ratios of P_4 and E_2 have been shown to be associated with early embryonic loss (Beard et al., 1994). Although P_4 inhibits $PGF_{2\alpha}$, low concentrations of P_4

are needed during late diestrus in order for sequestration of $PGF_{2\alpha}$ from the uterine endometrium (Vallet et al., 1990). It has been suggested that P_4 is important for the increase in PGHS activity which increases the rate of synthesis as well as stimulates PLA_2 for lipid accumulation in uterine epithelium for subsequent $PGF_{2\alpha}$ synthesis (Brinsfield and Hawk, 1973; Raw et al., 1988).

Heat Stress

Heat stress in dairy cattle causes many physiological changes, which may negatively affect fertility. Studies have shown that heat-stressed cows and heifers have reduced fertility and embryo development when compared to non-heat stressed cows (Ulberg and Burfening, 1967; Hyttel et al., 1986; Geisert et al., 1988; Putney et al., 1989; Ealy et al., 1993; Wolfenson et al., 1995 & 1997; Wilson et al., 1998a & 1998b; Schüller et al., 2014). One study reported that heat stressed cows on day 1 following fertilization produced embryos that had decreased development and overall viability, however by day 3 embryos became more heat resistant (Ealy et al., 1993). If heat stress causes a delay in embryonic development then IFNτ secretion may be delayed, thus failing to inhibit uterine $PGF_{2\alpha}$ synthesis and secretion during maternal recognition. In ewes, exposure to greater ambient temperatures ceased embryo development around 8 to 11 days after fertilization (Ulberg and Burfening, 1967). In both cows and heifers, heat stress caused prolonged luteal phases during the postpartum period (Wilson et al., 1998a & 1998b). This increase in the length of the luteal phase may delay subsequent ovulation, disrupt the estrus cycle, and lead to other issues such as failure to respond to synchronization programs. In cows, an increase in body temperature was associated with a decrease in P_4 concentrations and a disruption in conceptus development; however IFNτ secretion was not altered (Geisert et al., 1988). In

superovulated heifers, heat stress during the periovulatory period for 10 hours prior to the onset of behavioral estrus coincided with the early stages of oocyte maturation, causing 85% of embryos to not progress past 8 to 16 cells by day 7 (Hyttel et al., 1986; Putney, 1989). Heat stressed heifers had a greater occurrence of retarded and/or abnormal embryos that resulted in degenerative blastomeres. In fact heat stressed heifers only 12% exhibited normal embryos, whereas 68% of non-heat stressed heifers exhibited normal embryos (Putney et al., 1989). A study conducted by Schüller et al (2014) in lactating dairy cows indicated that when cows were heat stressed (73 vs. 41 temperature-humidity index; THI) from day 21 to 1 prior to AI there was a decrease in CR from 31% to 12%. These findings were similar to previous studies by Baumgartner and Chrisman (1981) where maternal heat stress negatively affected maturation of the oocyte prior to ovulation. Not only is the oocyte maturation negatively affected, studies have shown that heat stress reduces the number of follicles and growth of follicles prior to maturation (Wolfenson et al., 1995 & 1997). The authors noted that inhibition of follicular growth led to incomplete dominance of the ovulatory follicle and a reduction of E_2 and subsequent P_4 production by the CL (Wolfenson et al., 1995 & 1997). Reduction in steroid hormones (E_2/P_4) further support the fact that overall PR is negatively affected when dairy cows and heifers are heat stressed prior to AI. Collectively, these results indicate that maternal heat stress during oocyte development and maturation, as well as early embryo development decrease CR and may lead to early embryonic mortality.

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

Non-steroidal anti-inflammatory drugs (NSAIDS) have analgesic, antipyretic and anti-inflammatory effects and are used for therapeutic purposes for alleviation of pain, fever

and inflammation. Non-steroidal anti-inflammatory drugs are known inhibitors of COX enzymes, which regulate the conversion of AA to $PGF_{2\alpha}$ (Lees et al., 2004; Rao and Knaus, 2008; Agrawal and Gupta, 2010). Prostaglandins are important for vasodilation, erythema and hyperalgesia processes, as a result of injury or disease. A wide variety of NSAIDS such as flunixin meglumine (FM) and ibprofen are known COX inhibitors and have been shown to have reproductive benefits in livestock, mice and humans (Aiumlamai et al., 1990; Breuhaus et al., 1999; Rubinstein et al., 1999; Stahringer et al., 1999; Al Janabi et al., 2005; Scenna et al., 2005; Kafi et al., 2006; Guzeloglu et al., 2007). Veterinary practitioners either prescribing or administering NSAIDS for reproductive purposes must follow any extra-label drug use (ELDU) outlined in the Animal Medicinal Drug Use Clarification Act (AMDUCA) (Payne, 2001).

Non-steroidal anti-inflammatory drug metabolites bind to circulating albumin (with > 95% binding, except salicylate acid ~50%). This binding protein allows for the distribution of NSAIDS through circulation to various tissues as well as increases the half-life following drug administration (Agrawal and Gupta, 2010). Some adverse effects of NSAIDS include common gastrointestinal issues. The potency and therapeutic efficiency of NSAIDS depend on the type of NSAID used as well as the route of administration.

Ketoprofen

Ketoprofen is a propionic NSAID; a non-selective inhibitor of COX 1 and 2 (Agrawal and Gupta, 2010). Ketoprofen inhibits prostaglandin production providing analgesic, antipyretic, and anti-inflammatory effects. Ketoprofen is usually given to cattle for fever, pain and inflammation associated with mastitis because ketoprofen has no milk withdrawal period (Agrawal and Gupta, 2010). When ketoprofen was administered to dairy

cattle before and after ovulation, luteal regression was delayed and ketoprofen treated cattle had greater mean concentrations of P_4 when compared to control on days 0 to 6 of estrous cycle and greater E_2 concentrations compared to control cows at time of estrus (Kafi et al., 2006). This delay in luteal regression indicates that ketoprofen may also suppress synthesis and secretion of $PGF_{2\alpha}$. Given that luteal regression was delayed, the researchers noted that follicles that developed during ketoprofen administration had impaired growth and therefore negatively affected the subsequent cycle (Kafi et al., 2006).

Flunixin Meglumine

Flunixin meglumine is a potent COX inhibitor that elicits analgesic, antipyretic, and anti-inflammatory effects. Previous research has shown that FM also inhibits prostaglandin H_2 synthase ($PGHS_2$), which converts AA into PGH_2 (Anderson et al., 1990). In cattle, FM is commonly used for pyrexia and inflammation during bovine respiratory disease (BRD) and endotoxemia (Agrawal and Gupta, 2010). Flunixin meglumine should be given intravenously (i.v.) and has a milk withdrawal of 36 hours and a meat withdrawal of 4 days (Payne, 2001). It has been well documented that the use of FM can increase PR during embryo transfer in both beef and lactating dairy cattle (Schrick et al., 2001; Purcell et al., 2005). In beef cattle, the PR was increased by 12.7% when cows were treated with FM prior to embryo transfers of both fresh and glycerol-frozen embryos and there was also an association with the stage and quality of the embryo (Schrick et al., 2001).

The use of FM in dairy heifers prior to luteolysis of the CL improved PR on day 29 (FM 76% vs. control 50%) and day 65 (FM 69% vs. control 46%) following treatment (Guzeloglu et al., 2007). In lactating dairy cows, administration of FM decreased PGFM concentrations by 30% relative to the basal level and delayed luteolysis of the CL

(Aiumlamai et al., 1990). Another study in lactating dairy cows, also demonstrated that administration of FM significantly decreased PGFM concentrations, as well as maintained P_4 concentrations following treatment during the luteal phase of the estrous cycle concurrent with the time of embryo signaling (Ahmadzadeh et al., 2010). Similarly, a study by Pfeifer et al. (2007) demonstrated that FM administered 12 hours apart the evening of day 15 and morning of day 16 of the estrous cycle significantly improved PR (37% FM vs. 17% control) 30 days following AI in lactating dairy cows. The authors suggested that these observed results were due to the attenuation of $PGF_{2\alpha}$ secretion preventing early luteolysis of the CL (Pfeifer et al., 2007). In beef cows, administration of FM 14 days following AI increased PR/AI (71% FM vs. 61% control) compared to those that were not administered FM (Merrill et al., 2007). In this study, mean PGFM concentrations were significantly reduced following the administration of FM (39.6 pg/mL FM vs. 60.6 pg/mL control) similar to observations seen in lactating dairy cows (Merrill et al., 2007).

Although the above mentioned studies (Aiumlamai et al., 1990; Guzeloglu et al., 2007; Merrill et al., 2007; Pfeifer et al., 2007; Ahmadzadeh et al., 2010) in beef cows, dairy heifers and lactating dairy cows have demonstrated a decrease in PGFM and/or an increase in PR/AI, a few studies in dairy heifers have shown no effect of FM on overall PR/AI. A study by Rabaglino et al. (2010), observed no differences in PR/AI when FM was administered twice 12 hours apart on day 15 and 16 after AI in dairy heifers synchronized using a 5-day CIDR-Cosynch TAI protocol (59.4% FM vs. 59.5% control). Similarly, a study by von Krueger and Heuwieser (2010), showed no difference in PR/AI (54.8% FM vs. 58.2% control) when FM was administered to dairy heifers 24 hours apart on either day 14 and 15 or 15 and 16 post-AI.

Although administration of FM may slow or prevent luteolysis and increase PR in lactating cows, the use of FM is not practical. It requires a prescription from a veterinarian, is more expensive than other NSAIDS, and requires milk withdrawal of 36 hours resulting in milk profit loss (Payne, 2001). Collectively, the studies mentioned above indicate that NSAIDS can inhibit $PGF_{2\alpha}$ during the luteal phase of the estrous cycle, maintain P_4, and potentially improve fertility. However, alternative NSAIDS may prove to be just as effective in inhibiting secretion of $PGF_{2\alpha}$ and improve fertility without the limitation that are associated with FM.

Aspirin

Aspirin is carboxylic acid NSAID and is an inhibitor of COX enzyme. It is mostly used in the dairy industry for the treatment of pain, fever and inflammation (Agrawal and Gupta, 2010). Aspirin is known to inhibit prostaglandin synthase, which further reduces the production of prostaglandins (Vane, 1971). Aspirin is commonly administered orally in bolus form and contains lipophilic molecules, which allow for absorption through the gastrointestinal cells. However, only 50% of aspirin's metabolite (salicylate acid) binds to albumin for distribution and within 24 hours following administration, 90% of aspirin is eliminated through the urine via the glycine conjunction pathway (Short et al., 1991; Agrawal and Gupta, 2010).

In cattle, an effective dose of orally administered aspirin is 100mg/kg body weight (BW) every 12 hours, which is equivalent to 1 ⅓ of 240 grain (20.68 g) bolus twice a day for a 1500 lb cow (Gingerich, 1975; Payne, 2001). Aspirin has been shown to affect blood flow by shifting the local production of thromboxane and prostaglandins to prostacyclin (Elli et al., 2001). In sheep, administered 10mg/kg BW aspirin i.v. decreased the basal

thromboxane level by 95% (Nolan et al., 1990). Rubinstein et al. (1999) postulated that aspirin-induced reduction in thromboxane is the mechanism by which the smooth muscles of the uterine endometrial vessels relax, increasing blood flow to the uterus.

Administration of low dose aspirin in women, during embryo transfer for *in vitro* fertilization, has shown promising results to improve overall successful implantation and PR (Rubinstein et al., 1999). Administration of aspirin improved ovarian responsiveness, which was measured by the number of oocytes ovulated by an injection of human chorionic gonadotropin (hCG), an increase in uterine and ovarian blood flow, and improved implantation rates and PR (Rubinstein et al., 1999). In mice, administration of low dose aspirin, during diestrus over long periods of time (5 to 20 days), significantly decreased uterine weight and development, decreased serum levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH), as well as an increase in P_4 levels, and the number and diameter of CL's present on the ovary (Al-Janabi et al., 2005). However, in this study the researchers did not determine any fertility aspects such as number of pups or PR following aspirin treatment.

In Brahman cows (40 multiparous and 20 primiparous), 2.3 kg of aspirin was added to concentrate diet (approximately 100 mg/kg BW) and given every 12 hours for 5 days (day 7 to 13) postpartum to observe effects on PGFM and P_4 concentrations. In this study, multiparous cows showed a decrease in plasma PGFM concentrations whereas primiparous cows exhibited an increase in plasma PGFM in which the authors were unable to explain (Strahringer et al., 1999). Following aspirin treatment, both primiparous and multiparous cows were reported to have lower PR, increased abnormal cycles and a declined presence of a CL following ovulation (Strahringer et al., 1999). However, the length of the first estrous

cycle, concentrations of P_4, and conception interval (CI) were all unaffected following aspirin administration (Stahringer et al., 1999). Given that aspirin is less expensive and does not currently have a milk or meat withdrawal, its use, as a reproductive management tool may prove beneficial in lactating dairy cows to decrease premature $PGF_{2\alpha}$ secretion, and reduce early embryonic loss. Given the lack of evidence on the effects of oral administration of aspirin on PGFM and P_4 concentrations during the luteal phase of the estrous cycle in lactating dairy cows, justifies further investigation. Further research could determine any potential benefits of oral aspirin administration on the inhibition of premature $PGF_{2\alpha}$ secretion and the maintenance of P_4 during the time of embryo signaling.

OBJECTIVES AND RATIONALES

HYPOTHESIS

Non-steroidal anti-inflammatory drugs are inhibitors of COX enzymes, and inhibit the synthesis of prostaglandin. Although NSAIDS are commonly used for therapeutic purposes in the dairy industry, the use of these drugs may be beneficial in preventing the early secretion of $PGF_{2\alpha}$, premature regression of the CL, and therefore prevent early embryonic loss. Currently there is a lack of evidence on aspirins ability to decrease PGFM concentrations and sustain P_4 concentrations following administration during the late luteal phase of the estrous cycle in lactating dairy cows. Therefore, it is our hypothesis that oral administration of aspirin during days 14 and 15 of the estrous cycle may help prevent $PGF_{2\alpha}$ synthesis and secretion and maintain luteal function in lactating Holstein dairy cows.

OBJECTIVES

To examine the effects of orally administered aspirin during days 14 and 15 of the estrous cycle, on $PGF_{2\alpha}$ secretion and luteal function in lactating Holstein dairy cows. Specific goals:

1) Evaluate the effects of oral administration of aspirin on:
    a. Secretion of $PGF_{2\alpha}$
        i. By characterizing plasma PGFM concentrations.
    b. Describe post-treatment luteal function
        i. By characterizing plasma P_4 concentrations.

MATERIALS & METHODS

ANIMALS

This study was conducted in October 2012 and March 2013 at the University of Idaho Dairy Research and Education Center located in Moscow, Idaho. All animal handling procedures and treatment protocols were approved prior to initiation of the experiment by the University of Idaho Animal Care and Use Committee (ACUC) (Appendix 2). Twenty-five lactating Holstein dairy cows, approximately 30 days in milk (DIM), were synchronized to ovulation using a 5-day CIDR-Cosynch protocol (Bridges et al., 2008). All animals were housed in free stall barns and milked twice daily. Cows were fed a total mixed ration (TMR) with ad libitum access to both feed and water.

CHARACTERISTICS OF RESEARCH COWS

While initially, 25 cows were subjected to our study, one cow was eliminated at the time of serial blood sampling due to treatment with FM for illness. Another cow was

eliminated following analysis of P_4 concentrations and having < 1 ng/mL P_4 on day 15 of experiment (Figure 1.1) indicating that this cow was not synchronized to the luteal phase of the estrous cycle. All cows included in analysis were healthy at the initiation of the experiment (i.e no metabolic or postpartum disorders prior to trial) and endured no disease during this trial.

EXPERIMENTAL PROCEDURES

In order to bring all animals to the same stage of estrous cycle, cows were synchronized using a 5-day CIDR-Cosynch protocol. Briefly, on day -8, all cows were administered gonadotropin releasing hormone (GnRH; 100μg Factrel, i.m.; Fort Dodge Animal Health, Fort Dodge, IA), and received a controlled internal drug releasing (CIDR, 1.38 g P_4, Eazi-Breed CIDR, intravaginal; Zoetis, Florham Park, NJ) insert for 5 days. On day -3, CIDR inserts were removed and $PGF_{2\alpha}$ (25mg, Lutalyse, i.m.; Zoetis, Florham Park, NJ) was given to regress CL. Estrural behavior was monitored 4 times daily by observational ware of heat patches (Estrotect™; CRV, Ambreed NZ) placed upon the tail head. On day 0, all cows that did not show estrus were given GnRH (100μg) to cause ovulation of the dominant follicle (Figure 1.1). All injections were given using 18 gauge 1 ½" needles intramuscularly (i.m.) into the gluteus medius between the hooks and pins of the rump area.

Ultrasounds

All cows were subjected to transrectal ultrasonography (Aloka SSD-500 V, Aloka, Tokyo, Japan) on days 0, 3, and 7 in order to assess follicular dynamics and to determine ovulation and presence of CL. The location and number of CL's present were recorded. Ovulation was defined as the disappearance of any follicle >10mm in diameter and the

formation of a CL in the same location (Sellars et al., 2006). Any spontaneous regression of the CL allowed for determination of non-luteal phase cows, which were then eliminated from the study prior to initiation of treatment (Figure 1.1).

Treatment

Fourteen days following estrus detection or GnRH administration, and after determination of a CL, cows were stratified by parity (primiparous or multiparous) and randomly assigned to one of the two groups. The treatment group was orally administered 1 ½ (480 grain) bolus of aspirin (n = 11; 46.65 g/dose; Aspen Veterinary Resources® Ltd., Liberty, MO) 12 hours apart in the morning and evening of day 14 and the morning of day 15, while the control group (n = 12) received sham bolus administration. In addition, coccygeal blood samples were obtained for pretreatment PGFM concentrations quantification on day 14. On day 15, after the last dose of aspirin was administered in the morning and cows were moved into tie stalls. Six hours post-treatment, hourly coccygeal blood samples were collected for 6 hours to measure PGFM concentrations (Figure 1.1). Cows had access to a total mixed ration (TMR) and water ad libitum during the hourly blood collection. During the serial blood sampling on day 15, body condition scores (BCS) (scale of 1 to 5) (Edmonson et al., 1989) were assessed by averaging scores from three individual accessors. Following blood sampling, all cows were weighed and body weights (BW) were recorded before returning to the free stalls. Body weights and BCS were taken following treatment as all cows receiving aspirin received the same dosage of aspirin and these response variables were recorded in order to determine any differences between treatment groups that may have explained variable results. For eight days following treatment (day 15 to 22), daily blood samples were collected for analysis of P_4 and to assess luteal function and

determine day of luteolysis. The day of luteolysis was defined by two consecutive days of <1 ng/mL of P_4.

Milk yield was recorded for three randomly selected days prior to the initiation of the experiment, and three days during the experiment to determine any deleterious effects. Days in milk, parity and pen assignments were recorded and analyzed for any possible confounding effects.

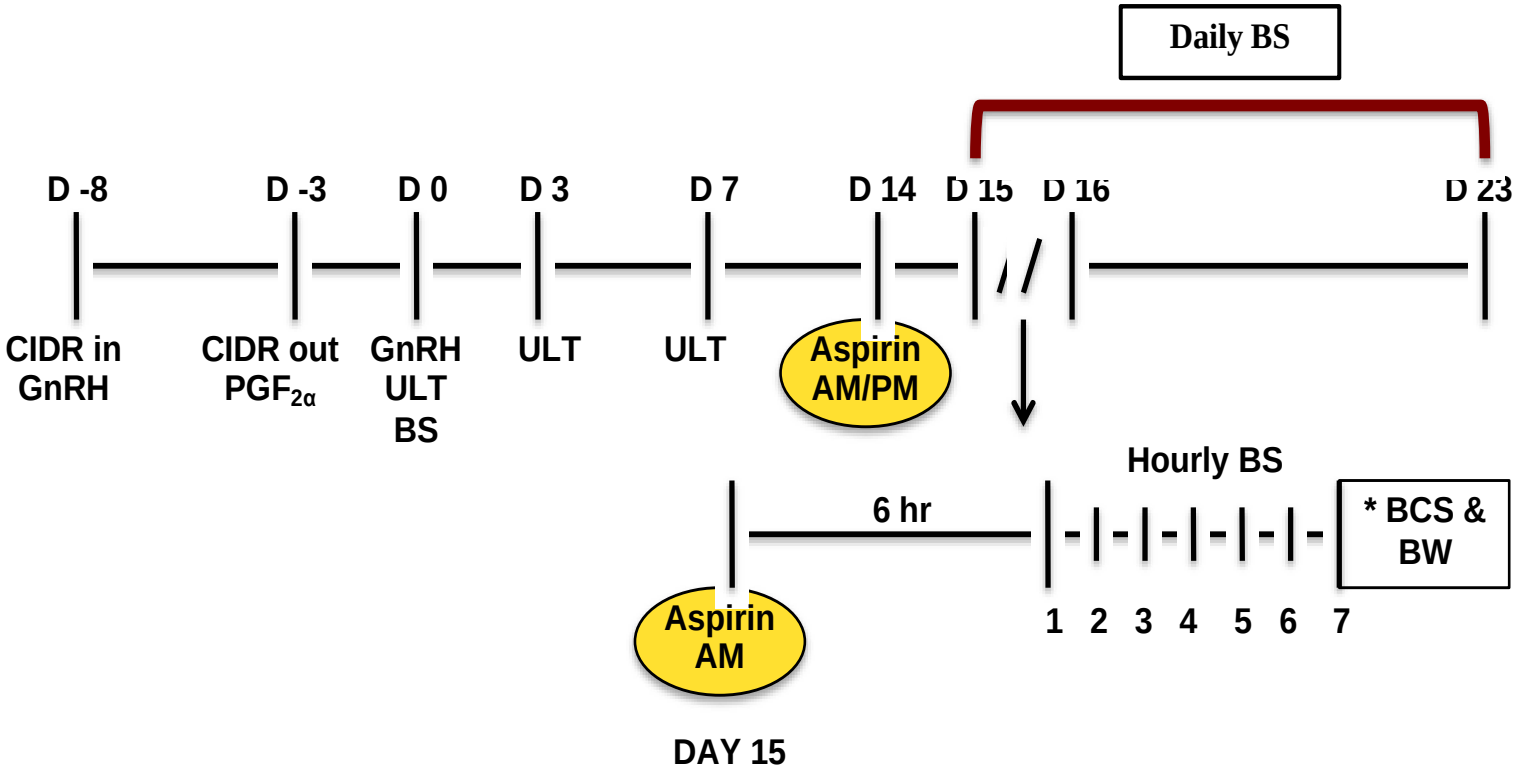


Figure 1.1 Experimental protocol, including estrous synchronization and treatment during the luteal phase of the estrous cycle in lactating Holstein cows. Aspirin treatment included 46.65 g/dose 3x every 12 hours. Serial coccygeal blood samples were collected 6 hours after last dose of aspirin for 6 consecutive hours on day 15 to determine prostaglandin metabolite (PGFM) concentrations. Daily coccygeal blood samples were taken from day 15 to 22 to determine progesterone (P_4) concentrations and monitor day to luteolysis (two consecutive days of < 1ng/mL P_4).

Blood Collection and Processing

Blood samples were collected on day 0 and from days 15 to 22 (8 samples/cow) for P_4 concentration. Blood samples collected on day 14 and for 6 hours on day 15 (8 samples/cow) were analyzed for PGFM concentrations (Figure 1.1). All blood samples were collected via coccygeal venipuncture. The proximal ventral surface of the tail was sanitized using 70% ethanol, then the tail was punctured using a 20 gauge 1 ½" single use blood collection needle. Blood samples were collected using 10 mL 15% EDTA (K_3) Kendell Monoject Vacutainers® (Tyco, Mansfield, MA), labeled and then immediately placed on ice. Within one hour all samples were centrifuged for 12 minutes at 2500 x g at room temperature. Plasma was harvested and then samples were stored at -20^o C until assayed for PGFM and P_4. On day 15 after each hourly blood sample was harvested for plasma, 1 mL of plasma was aliquoted into conicle tubes for the analysis of PGFM in order to prevent freeze-thaw damage.

Prostaglandin Metabolite Assay

Serial blood samples from day 15 were assessed for PGFM concentrations using an ELISA assay (Cayman Chemical kit 13,14-dihydro 15-keto $PGF_{2\alpha}$) as previously described by Del Vecchio et al. (1992). The assay standard curve ranged from 2.3 to 5,000 pg/ml, and all samples were assayed in duplicate. Samples were diluted 1:1 with enzyme immunoassay (EIA) buffer in order to reach the 20% to 80% detection limit on the standard curve for better accuracy of PGFM concentrations. Once samples were loaded into 96-well plates, they were placed at 4°C for 18 hours. Subsequently, color density of each sample was determined using a spectrophotometer read at 412 nm.

The standard curve was plotted using the following equation:

$$\text{Logit } (B/B_0) = \ln [B/B_0/(1-B/B_0)]$$

B = percent bound/maximum bounding

B_0 = maximum binding

The inter-assay coefficients of variance (CV) were 30.6%, between 6 plates, respectively.

Progesterone Assay

Day 0 blood samples were analyzed for P_4 concentrations in order to determine that all cows were synchronized to the follicular phase. Daily blood samples from days 15 to 22 were analyzed for P_4 concentrations to insure cows were in the luteal phase of the estrous cycle and to determine day of luteolysis (Figure 1.1). Progesterone concentrations were quantified using a solid-phase radioimmunoassay (RIA; Siemens Corp., Los Angeles, CA). The standard curve ranged from 0.1 to 40 ng/mL and the standard curve and all samples were run in duplicate. The intra-assay coefficient of variance (CV) was 7.8%.

Day of luteolysis was defined by two consecutive days of < 1 ng/mL P_4. Seven cows (4 control and 3 aspirin) were eliminated from analysis, as they never experienced luteolysis as described by our definition. Therefore for this analysis 16 cows (8 aspirin and 8 control) were analyzed for the day at which luteolysis occurred.

STATISTICAL ANAYLSIS

To determine the differences between treatment groups for response variables BW, BCS, DIM, parity (primiparous vs. multiparous) and milk production, analysis of variance procedures were utilized. The model included treatment, parity and treatment x parity interactions. Analysis of variance procedures were also used to determine the difference in

P_4 concentrations between the two treatment groups on day 0 of the experimental protocol (Appendix 1.1), as well as effects of treatment (aspirin vs. control) on day to luteolysis (Appendix 1.2).

Analysis of repeated measures using the mixed model procedure Autoregressive Moving Average (ARMA; 1,1) was used to determine differences in PGFM and P_4 concentrations between treatment groups. The model included treatment effects, the repeated measure factor, time, and the interactions between treatment and time (Appendix 1.3 & 1.4). The random effect in this model was cow (subject) within treatment. Also, BW was incorporated into the PGFM models as a covariate (Appendix 1.4). All statistical computations were carried out using SAS version 9.3 (2011). Statistical significance was declared at a $P < 0.05$, and a tendency at $P = 0.1$.

RESULTS

RESPONSE VARIABLES

Twenty-three primiparous and multiparous lactating Holstein cows were used in this study. Cows were stratified by parity and randomly assigned to treatment (aspirin; n = 11) or control (no aspirin; n = 12). There was no differences ($P > 0.05$) in BW (561.8 vs. 541.8 ± 17 kg, aspirin vs. control), DIM (34 vs. 36 ± 6 days), BCS (2.5 vs. 2.7 ± 0.07), and milk production (37.7 vs. 35.4 ± 1.8 kg) between treatment groups (Table 1.1). As expected, BW differed between multiparous and primiparous cows (613.6 vs. 490.4 ± 17 kg, $P < 0.01$). Also, milk production tended to differ between parity groups (39 vs. 34 ± 1.8 kg, $P = 0.08$). However, there was no effect of parity on DIM and BCS.

Table 1.1 Mean ± SE body weight (BW), days in milk (DIM), body condition score (BCS), and milk yield (Milk) in lactating Holstein cows in aspirin (n = 11) and control (n = 12) treatment groups.

Treatment	BW	DIM[1]	BCS[2]	Milk[3]
Aspirin[4] (n = 11)	561.8 ± 15.6 kg	34 ± 6	2.5 ± 0.07	37.7 ± 1.9 kg
Control (n = 12)	541.8 ± 15.1 kg	36 ± 6	2.7 ± 0.07	35.4 ± 1.8 kg

[1] DIM on day -8 of experimental protocol.
[2] BCS on scale of 1 to 5 in 0.25 increments (1=emaciated; 5=over conditioned).
[3] Milk Production of 6 randomly selected days, 3 prior to and 3 during experiment.
[4] Aspirin was administered orally, total dosage of 140 g.

ULTRASONOGRAPHY

Ovarian structures were recorded and mapped using transrectal ultrasonography on days 0, 3 and 7. All cows included in the data did not show estrus prior to day 0 and presented a dominant follicle at the time of GnRH administration (day 0). On day 3 and 7, ovulation was confirmed by the absence of an ovulatory follicle (> 10 mm in diameter) and the development of a CL in the same location as the ovulatory follicle. All 23 cows had a CL at the initiation of treatment.

PROSTAGLANDIN METABOLITE

The PGFM results were skewed indicating an unstable variance with a non-normal distribution. Therefore, the Proc Univariate procedure in SAS was performed and confirmed a large variation (Appendix 1.5). The PGFM data was transformed using the LN function in order to stabilize the variance and control non-normal distribution of data as it is a monotonic transformation and maintains integrity of the data (Appendix 1.5).

The appropriate dose of aspirin in cattle is 100mg/kg BW every 12 hours (Gingerich, 1975). Provided that all cows regardless of BW received 1 ½ bolus (480 grains) three times 12 hours apart (140 g total), BW was used as a covariate in the model to assess any effects of BW on PGFM concentrations following aspirin administration. There was no significant difference ($P = 0.12$) of BW on PGFM concentrations, however BW was left in the model for PGFM analysis as a covariate to help explain some of the residual error.

The mean PGFM concentration before initiation of treatment (day 14) did not differ between aspirin and control groups (5.1 vs. 5.2 ± 0.2; $P > 0.05$; Figure 1.2 & 1.3). Overall, there was an effect of treatment and treatment × time on mean PGFM ($P < 0.05$; Figure 1.2). Mean PGFM concentrations decreased ($P < 0.05$) 30 h after the first dose of aspirin, and remained low for 11 h after the last dose of aspirin, whereas PGFM in the control remained unchanged (Figure 1.2). The mean PGFM concentrations were 4.3 ± 0.19 for aspirin and 4.9 ± 0.18 for control ($P < 0.05$; Figure 1.4).

Estimated least squared (LS) means (based on log transformation) of plasma PGFM concentration in aspirin-treated and control cows during the experimental period is depicted in Figure 1.3. Throughout the experimental period, mean PGFM concentrations remained below 80 pg/mL in aspirin-treated cows, whereas in the control group mean PGFM concentrations remained above 120 pg/mL (Figures 1.3 & 1.4). Mean PGFM concentrations was decreased by approximately 1.87 fold in aspirin-treated cows compared to control (Figure 1.4).

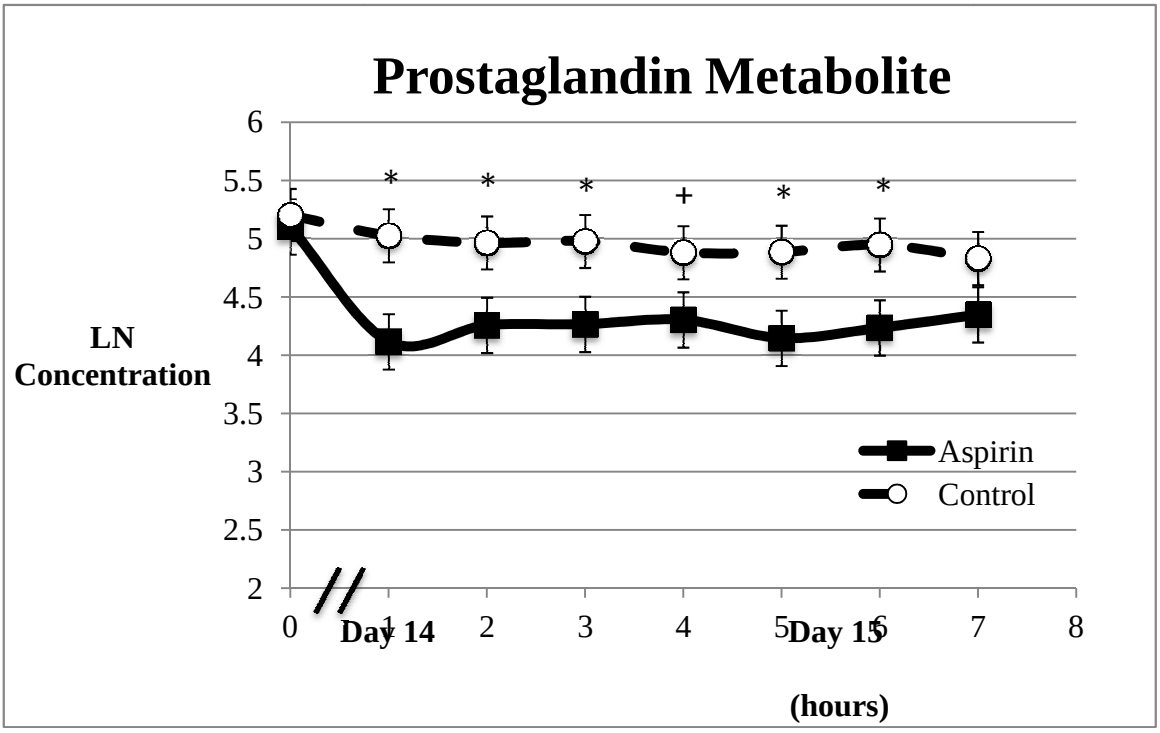


Figure 1.2 Mean prostaglandin metabolite (PGFM) concentrations, after data transformation, between aspirin (n = 11; 140 g total) and control (n = 12) groups of lactating Holstein dairy cows during the luteal phase of the estrous cycle. Time 0 is considered day 14 prior to first aspirin bolus administration. Hourly blood samples were taken for 6 consecutive hours after last aspirin treatment.

* Means differ from aspirin-treated cows ($P \leq 0.05$).
+ Means differ from aspirin-treated cows ($P = 0.08$).

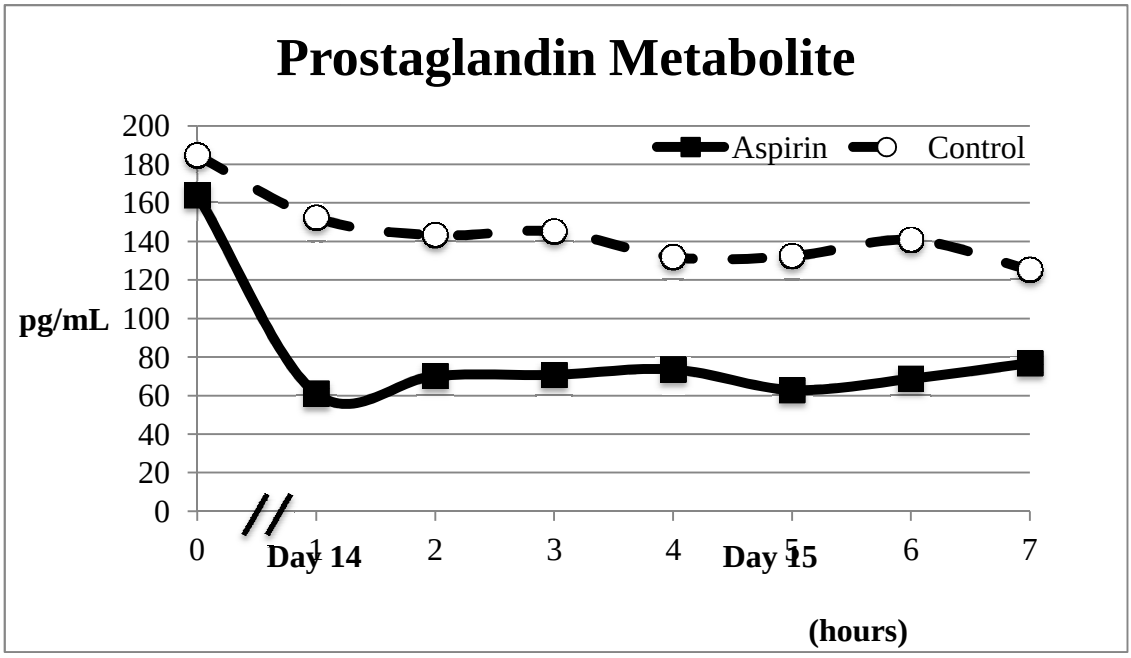
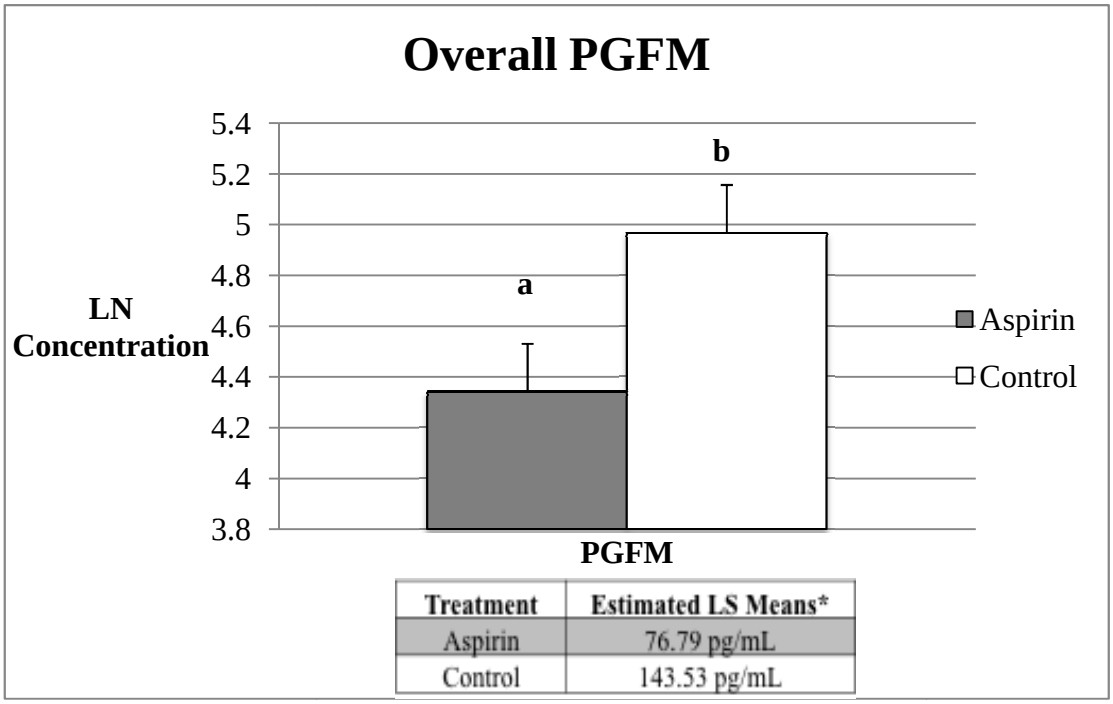


Figure 1.3 Estimated Least Squared (LS) means* of plasma prostaglandin metabolite (PGFM) concentration in aspirin (n = 11; 140 g total) and control (n = 12) in lactating Holstein dairy cows during the luteal phase of the estrous cycle. Time 0 is considered day 14 prior to first aspirin bolus administration. Hourly blood samples on day 15 were taken for 6 hours following last bolus administration. Concentrations of PGFM are given in pg/mL.

* = Estimated LS means based on log transformed PGFM.



Treatment	Estimated LS Means*
Aspirin	76.79 pg/mL
Control	143.53 pg/mL

Figure 1.4 Mean transformed prostaglandin metabolite (PGFM) concentrations on day 15 serial blood samples between aspirin (n = 11; 140 g total) and control (n = 12) groups of lactating Holstein cows during the luteal phase of the estrous cycle. Estimated least squared (LS) mean* concentrations of PGFM are given in pg/mL in table below graph.

ab Bars with different letters differ ($P < 0.05$).

* Estimated LS means based on log transformed PGFM.

PROGESTERONE

There was no effect of treatment (P = 0.66) or treatment by day (P = 0.70) on mean P_4 concentrations. Daily blood samples from day 15 to 22 were on average 3.6 ± 0.6 ng/mL for aspirin and 3.2 ± 0.6 ng/mL for control groups. However, there was an effect of day on mean P_4 concentrations (P < 0.01), as both groups mean P_4 concentrations steadily decreased over time (Figure 1.5).

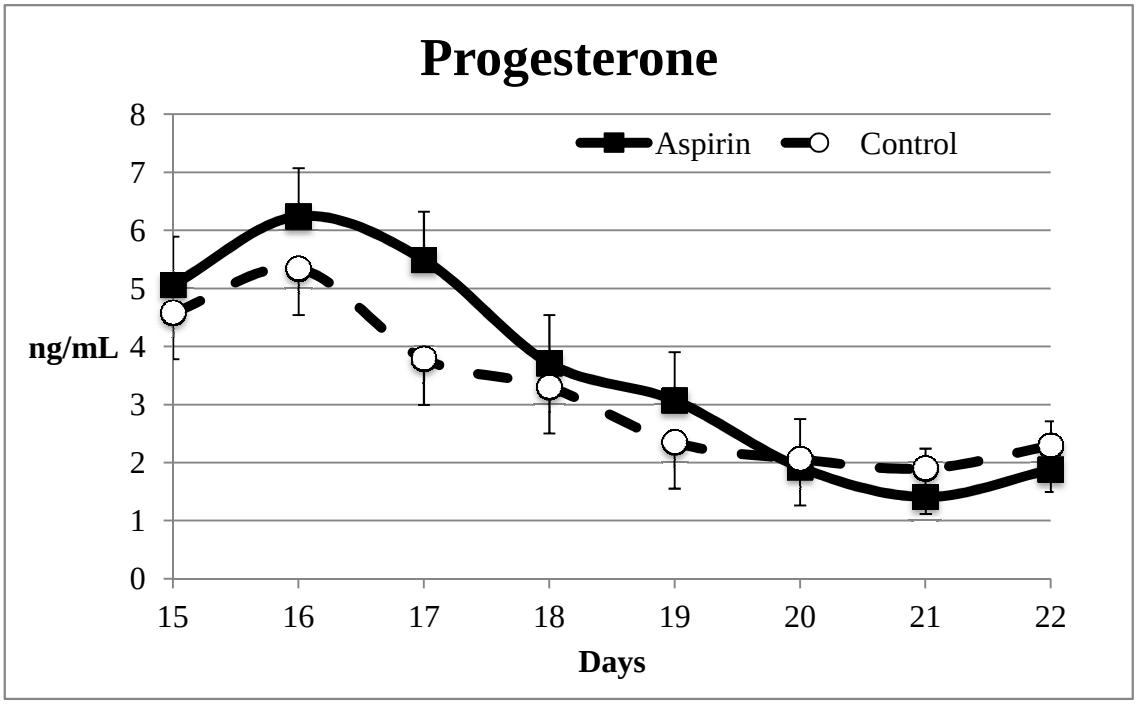


Figure 1.5 Mean progesterone (P_4) concentrations in aspirin-treated (n = 11; 140 g total) and control (n = 12) lactating Holstein cows from day 15 to 22 of experimental protocol. Day 0 is considered to be the day of the final gonadotropin-releasing hormone (GnRH) during experimental protocol.

DAY TO LUTEOLYSIS

Data from 16 cows were used for the analysis of day to luteolysis. Seven cows (4 control and 3 aspirin) were eliminated from this analysis, as they did not experience luteolysis and P_4 concentrations never reached below 1 ng/mL. Day to luteolysis as previously described, was the day in which two consecutive daily blood samples had P_4 concentrations of < 1ng/mL. Interestingly, there was a tendency (P = 0.09) of day to luteolysis to differ between aspirin and control groups. Luteolysis occurred approximately a day later in aspirin-treated cows, and days to luteolysis were 5.6 ± 0.5 days (from day 15 of the experiment) for aspirin and 4.25 ± 0.5 days for control (Figure 1.6), respectively.

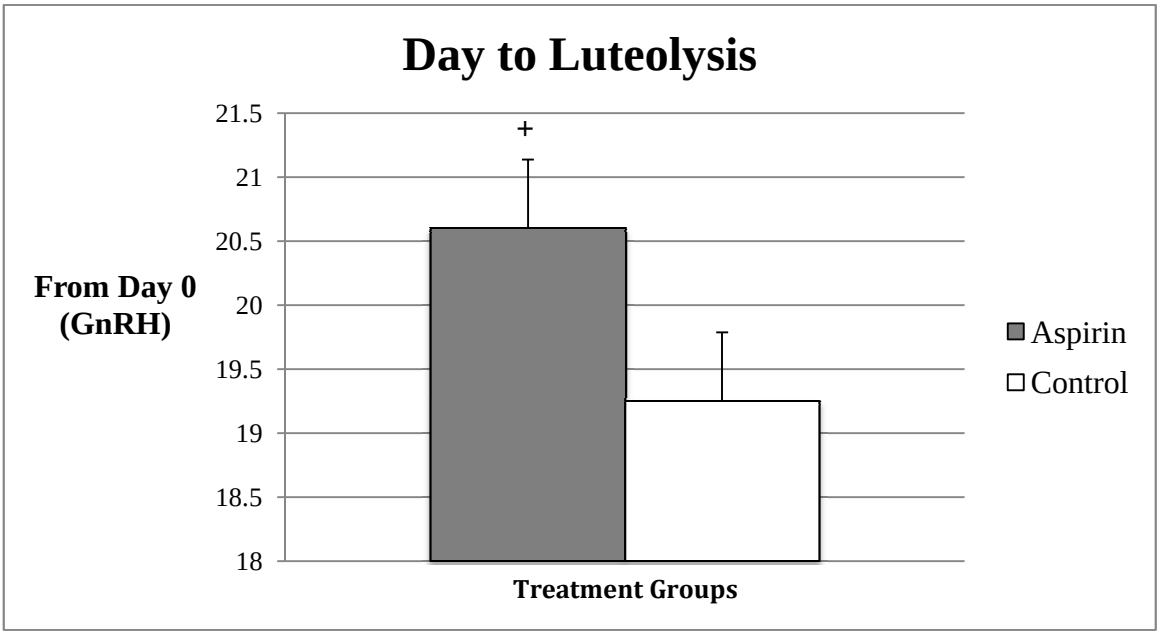


Figure 1.6 Mean day to luteolysis in lactating Holstein cows in aspirin (n = 8) and control (n = 8). Day of luteolysis was determined by two consecutive daily blood samples in which progesterone (P_4) was < 1 ng/mL. Days are represented by day of estrous cycle, where day 0 is the final gonadotropin-releasing hormone (GnRH).

+ Means tended to differ from the control group (P = 0.09).

DISCUSSION

Aspirin is primarily used in dairy cows for therapeutic purposes; however, aspirin as an NSAID may be a useful reproductive tool in preventing premature $PGF_{2\alpha}$ secretion during the estrous cycle. The main objectives of this study were to determine the effects of aspirin on $PGF_{2\alpha}$ secretion by characterizing plasma PGFM concentrations and luteal function by characterizing P_4 concentrations in lactating Holstein cows during the luteal phase of the estrous cycle.

Prostaglandin metabolite concentrations are stable metabolites of $PGF_{2\alpha}$, nevertheless, blood PGFM concentrations account for all circulating prostaglandins. However, studies in both ovine and bovine species have shown that PGFM concentrations are correlated with uterine-ovarian $PGF_{2\alpha}$ concentrations, and therefore can be used to assess $PGF_{2\alpha}$ concentrations (Kindahl and Basu, 1976; Mitchell et al., 1976; Kindahl et al., 1981; Thatcher et al., 1984; Zollers et al., 1993). Collectively these studies concluded that peripheral PGFM can be used as an index for uterine prostaglandin secretion and is a valid method for measuring $PGF_{2\alpha}$ secretion.

Our results provide the first evidence that administration of aspirin during the luteal phase of the estrous cycle (on day 14 and 15) in lactating Holstein dairy cows decreased plasma PGFM (Figure 1.3). Mean PGFM concentrations did not differ ($P > 0.05$) between aspirin and control groups on day 14 prior to administration of aspirin (Figure 1.2 & 1.3). Following last aspirin administration, on day 15 of the experimental protocol, there was a treatment and treatment by time effect on PGFM concentrations (Figure 1.2 & 1.3). Overall, mean PGFM concentrations were significantly ($P < 0.05$) lower for the aspirin than control

groups (Figure 1.4), and decreased between day 14 and 15 ($P < 0.01$; Figure 1.2 & 1.3) following aspirin administration.

We hypothesized that following aspirin administration, luteal function would be sustained which would be reflected by the maintenance of P_4 concentrations between days 15 and 22 of the experiment. There were no effects of treatment or treatment by time interactions on P_4 concentrations ($P > 0.05$). However, as we expected both groups significantly decrease ($P < 0.01$) P_4 concentrations over time as regression of the CL occurred (Figure 1.5). Although we did not observe a difference in P_4 concentrations between the aspirin and control groups (Figure 1.5), there was a tendency ($P = 0.09$) for the aspirin group (20.6 ± 0.5 days) to have longer days to luteolysis compared to the control group (19.25 ± 0.5 days) (Figure 1.6). This observation possibly indicates that the delay in day of luteolysis may be associated with a decrease in PGFM secretion.

In our study, the administration of oral aspirin on PGFM and P_4 concentrations were similar to previous findings in early postpartum Brahman cattle (Stahringer et al., 1999) in which aspirin treated cows showed a decrease in PGFM concentrations while no difference was observed in P_4 concentrations following administration of aspirin. In Stahringer et al. (1999) lactating Brahman cows (40 multiparous and 20 primiparous) were administered 100 mg/kg BW of aspirin (2.3 kg of concentrate diet) every 12 hours for 5 days, from days 7 to 13 postpartum, to observe its effects on reproduction and therapeutic effects postpartum. In the study conducted by Stahringer et al (1999) only multiparous Brahman cows decreased in PGFM, while primiparous cows increased PGFM concentrations following aspirin treatment. However, there was no clear explanation for this observation in that study. However, the results from lactating Brahman cows differed from our study, in which aspirin

treatment decreased PGFM concentrations in both primiparous and multiparous cows. Regarding P_4 concentrations following aspirin treatment our results were also similar to Stahringer et al. (1999) in that no differences in mean P_4 concentrations were observed following aspirin administration.

Early embryonic loss may in part be due to a delay in IFNτ secretion from the embryonic trophectoderm or the premature secretion of $PGF_{2\alpha}$ during maternal recognition (Thatcher et al., 1994; Inskeep, 2004). A lack of or untimely secretion of maternal recognition (later than day 15 or 16 post-fertilization) by IFNτ may lead to a decrease in P_4 and an increase in E_2 concentrations (Zollers et al., 1993; Thatcher et al., 1994; Inskeep, 2004). These changes in hormone profiles maintain OXY secretion from the ovary, which is then followed by an increase in circulating PGFM (Beard et al., 1994). High P_4 concentrations inhibit $PGF_{2\alpha}$ secretion, however elevated P_4 concentrations are also important for priming the uterine endometrium for $PGF_{2\alpha}$ synthesis by increasing PGHS and stimulating lipid droplet accumulation (Brinsfield and Hawk, 1973; Raw et al., 1988; Vallet et al., 1990).

The use of aspirin may potentially mimic the events of early pregnancy by abolishing early pulsatile secretion of prostaglandin. We observed a reduction in basal plasma PGFM concentrations by 62.8% (164.02 pg/mL on day 14 to 60.96 pg/mL six hours after last dose on day 15) when aspirin was administered on day 14 and 15 of the estrous cycle in lactating Holstein dairy cows (Figure 1.3). Although we did not observe maintenance of P_4 concentrations, days to luteolysis was delayed in the aspirin treated group indicating that CL regression may have been prolonged due to aspirin treatment. One basic question that arouse from this study was whether the blockage of pulsatile secretion of PGFM would

disrupt prostaglandin synthesis long-term following aspirin administration. In our study, approximately 11 hours following last aspirin administration PGFM concentrations became similar between treatment groups (Figure 1.2 & 1.3). This indicates that the uterus may not be affected long-term by aspirin treatment, however further research is needed to verify. From this study it can be concluded that aspirin treatment may be used to effectively decrease prostaglandin production and secretion by inhibiting COX enzymes, as our results indicated a decrease in plasma PGFM (Figure 1.2, 1.3 & 1.4) and a tendency ($P = 0.09$) for a delay in day to luteolysis (Figure 1.6) following aspirin administration.

The effect of feeding aspirin on fertility cannot be derived from the current study. Stahringer et al. (1999) reported that cows fed aspirin had a lower PR, increased abnormal estrous cycles, and a decline in the number of CL presence after estrus. However, in Stahringer et al. (1999) study, the first estrous cycle length and conception interval (CI) were not different between aspirin and control groups and breeding occurred 6 weeks after last aspirin treatment (Stahringer et al., 1999). The observed negative effect on PR could potentially be related to time of aspirin administration (day 7 to 13 postpartum), duration (5 days), route of administration (aspirin added to diet), or dosage (2.3 kg of concentrate diet) (Stahringer et al., 1999). In contrast, Al-Janabi et al. (2005) administered low dose aspirin long term to mice for 5, 10, 20 and 30 days and reported an increase in the number of CL, concentration of P_4, and a decrease in gonadotropins (LH/FSH). However some of the differences between these two studies can be explained by species differences.

This experiment along with Stahringer et al. (1999) suggests that aspirin acts in a similar manner as other NSAIDS such as FM. However, flunixin meglumine is administered i.v., causing a quicker biological action. Furthermore, it has been shown that

FM has a much higher potency than aspirin, which may be attributed to route of administration (Agrawal and Gupta, 2010). In previous studies, FM has been shown to decrease both PGFM circulating concentrations as well as maintain P_4 concentrations following treatment of FM in lactating dairy cows (Aiumlamai et al., 1990; Ahmadzadeh et al., 2009). Furthermore, in some studies, PR was improved following AI after lactating dairy cows and beef cows either treated with FM 2 to 12 minutes before embryo transfer or immediately following embryo transfer (Purcell et al., 2005; Scenna et al., 2005). Also in dairy heifers, FM administration on day 15 and 16 following fertilization improved PR (Guzeloglu et al., 2007).

Flunixin meglumine seems to have the potential to improve PR/AI when administered during the luteal phase of the estrous cycle. However, given the differences in route of administration and meat and milk withdrawal associated with administration, it is advantageous to explore alternative NSAIDS, which may provide similar results (Payne, 2001). In fact FM administration in beef cows, dairy heifers and lactating dairy cows, during the luteal phase of the estrous cycle has shown to decrease PGFM concentrations, maintain P_4 concentrations and increase PR/AI (Aiumlamai et al., 1990; Guzeloglu et al., 2007; Merrill et al., 2007; Pfeifer et al., 2007; Ahmadzadeh et al., 2009). However, other studies have shown no difference in PR/AI when FM was administered 14-16 days following TAI in dairy heifers (Rabaglino et al., 2010; von Krueger and Heuwieser, 2010).

As indicated the effect of FM administration on PR in cattle is not consistent. Moreover, FM requires a prescription from a licensed veterinarian and is associated a meat and milk withdrawal (Damian et al., 1997). In comparison between FM and aspirin, aspirin may be a more useful tool in suppressing PGFM concentrations and possibly delaying day

of luteolysis. Aspirin may also be a more feasible method from a management standpoint in preventing early embryonic loss. These benefits may be more likely observed in management or environmental conditions (e.g. during heat stress) that are conducive to early embryonic loss. It is well established that during summer months there is an increase in embryonic loss as well as impaired luteal function following GnRH (Geisert et al., 1988; Putney et al., 1989; Pursley et al., 1995; Vasconcelos et al., 1997). By preventing early embryonic loss, dairy producers may be able to improve PR/AI. However if developmental issues occurred during early embryo development that result in abnormalities or deformities, the prevention of embryonic loss may not be favorable as it would not result in a viable calf. Further research is needed to obtain the optimal dosage and length of aspirin administration in order to observe possible benefits on fertility under harsh conditions such as heat stress.

CONCLUSION

Early embryonic loss accounts for 70 to 80% of total embryonic loss and occurs between day 8 and 16 following fertilization. Maternal recognition by IFNτ inhibits the synthesis and secretion of $PGF_{2\alpha}$ by the uterine endothelium. The time period for maternal recognition is relatively short and it must occur during day 14 to 16 following fertilization to prevent $PGF_{2\alpha}$ secretion. Untimely secretion of IFNτ may allow for an increase in pulsatile release of $PGF_{2\alpha}$. Premature secretion of $PGF_{2\alpha}$ causes early luteolysis of the CL and may be one of the causes for early embryonic loss.

Prostaglandin $F_{2\alpha}$ is synthesized by the oxidation of AA by COX 1 and 2 enzymes. Therefore by inhibiting COX enzymes there is a potential to inhibit $PGF_{2\alpha}$ secretion. Non-steroidal anti-inflammatory drugs are known inhibitors of COX enzymes. The use of NSAIDS are mainly therapeutic, however the use of NSAIDS may be beneficial in

preventing premature secretion of $PGF_{2\alpha}$ which may result in early embryonic loss in lactating Holstein dairy cows. The results of the current study indicate for the first time that oral administration of aspirin during day 14 to 15 of the estrous cycle suppresses PGFM and may delay luteal regression in lactating dairy cows. However, further studies are required to determine the effects of aspirin on embryonic loss and fertility in lactating dairy cows.

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CHAPTER TWO

"Reproductive Performance in Dairy Cattle Following CIDR-Based Resynchronization: Influence of Administration of GnRH at the Initiation of the Protocol"

ABSTRACT

Approximately 60% of lactating Holstein dairy cows fail to conceive during the first artificial insemination (AI) and therefore must be reinseminated. One method of resynchronization is an Ovsynch protocol, with or without a controlled internal drug releasing insert (CIDR). However, pregnancy rates per AI (PR/AI) results from a Resynch-Ovsynch protocol have been inconsistent. Currently, there is a need for the development of an effective resynchronization protocols to improve pregnancy rate, reduce days open, and enhance production efficiency. The objective of this study was to determine the effect of the initial gonadotropin-releasing hormone (GnRH) administration on pregnancy rate per AI (PR/AI) in lactating Holstein dairy cows subjected to a 5-day CIDR-Cosynch resynchronization protocol after the first AI. Approximately 37 days after the first AI and upon non-pregnancy diagnosis, lactating cows eligible for a second AI (n = 429) were enrolled into a 5-day CIDR-Cosynch breeding protocol. On day 0, all cows received CIDR inserts and were assigned randomly to treatment (no GnRH; n = 203) or control (GnRH; 100 μg; n = 226) groups. Five days later, CIDR inserts were removed and all cows received one injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 500 μg). From day 5 to 8, cows were monitored daily for detection of estrus and either bred early (day 6 or 7) and not given GnRH or bred timed artificial insemination (TAI; day 8) and received a second GnRH (100 μg) injection. Blood samples were collected from a sub-group of cows (n = 184) on day 0 and analyzed for

progesterone (P_4) concentrations. There were no effects of treatment (no GnRH vs. initial GnRH, 27.09 % vs. 21.23 %, respectively) on PR/AI. There were also no effects of day of insemination (day 6 or 7 vs. day 8 of experimental protocol), or sire (n = 10), or any two-way interactions with treatment on overall PR/AI ($P > 0.05$). There was a tendency ($P = 0.06$) of technician (n = 3) effects as well as a significant ($P < 0.05$) difference between primiparous and multiparous on PR/AI. Mean P_4 did not differ between treatment groups (3.96 ± 0.34 ng/mL control vs. 4.51 ± 0.35 ng/mL treated) at initiation of experiment. Progesterone concentrations of animals in a sub-group (n = 184) were divided into two categories: high (≥ 1 ng/mL) and low (< 1 ng/mL). Overall PR/AI tended ($P = 0.09$) to be greater for high P_4 concentrations (n = 136) compared to low P_4 concentrations (n = 48) (26% vs. 16%, respectively). Collectively, these results indicate that the initial GnRH in a 5-day CIDR-Cosynch resynchronization program may not be necessary to achieve the same PR/AI, ultimately saving producers $2.50 to $3.50 per cow on reproductive management costs.

Keywords: dairy cows, resynchronization, 5-day CIDR-Cosynch

INTRODUCTION

Reproductive inefficiency can negatively affect farm profitability. In the past 25 years, pregnancy rates (PR) have steadily declined (Butler, 1998; Royal et al., 2000; Sreenan et al., 2001; de Vries and Risco, 2005). Approximately 90% of Holstein dairy herds in the US have < 20% PR (Durkin, 2010). This has led to an increase in average number of days open (DO). It has been reported that the average DO for lactating Holstein dairy cows in the US is 135 (± 67 days; n = 3,401,130 cows) and Idaho is 134 (± 66 days; n = 71,095 cows)

(Pszczola et al., 2009). An increase in PR has a substantial impact on dairy profitability. According to Overton (2005 & 2006), for every 1% increase or decrease in PR there is an average gain or loss of $18 to $25.

The average voluntary waiting period (VWP) is 50 to 70 days postpartum (Pursley et al., 1997; Caraviello et al., 2006). This period of time allows for uterine repair and involution as well as postpartum anestrus. Postpartum anestrus is defined as lack of cyclicity due to no ovulatory follicle present after parturition as well as insufficient GnRH from the hypothalamus to cause ovulation (Senger, 2012). As P_4 declines postpartum, 17β-estradiol (E_2) from the follicle rises and has a positive feedback on the hypothalamus surge center, which stimulates cyclicity (Senger, 2012). Studies have indicated that 6 to 59% of high producing Holstein dairy cattle 60 days postpartum will still not be cyclic (Moreira et al., 2000; Cerri et al., 2004; Stevenson et al., 2007; Santos et al., 2009). The lack of cyclicity could be due to various factors such as nutrition, lactation and increased steroid metabolism delaying resumption of ovarian activity (Wiltbank et al., 2006; Senger, 2012). Given the high proportion (6 to 59%) of high producing cows that are non-cyclic when first synchronization and AI occurs, estrous synchronization has become increasingly important in order to stimulate ovarian activity and maximize PR/AI.

In the United States, the majority (87%) of dairy producers use some methods of estrous synchronization for first AI (Caraviello et al., 2006). However, it has been reported that 55 to 65% of lactating dairy cows subject to the first postpartum AI will fail to conceive (Bisinotto et al., 2010). One of the main factors that contribute to this failure in conception to first AI is low heat detection (HD) efficiency rates, in fact approximately 50% of estrous periods will go undetected by producers (Nebel et al., 2000). Estrous synchronization

programs with TAI allows producers to synchronize all cows to similar stages of the estrous cycle, concentrating estrus expression into a short period of time. This allows for reduced labor costs as well as a potential increase of estrus detection, thus potentially improving conception rates (CR) (Pursley et al., 1997; Nebel and Jobst, 1998; Stevenson et al., 1999). Regardless of breeding protocol, it is well documented that pregnancy to first AI is usually less than 40% and therefore, 60% of cows will be subjected to a second AI (Pursley et al., 1997; Jobst et al., 2000; Fricke et al., 2003; Cerri et al., 2004; Chebel et al., 2006). Moreover, cows that do not conceive may have AI intervals longer than two estrous cycles if non-pregnancy status is not diagnosed and resynchronization protocols are not implemented in a timely manner (Dewey et al., 2010).

Provided that estrus expression and detection is poor (< 50%) across many dairy herds, synchronization of ovulation and TAI protocols are valuable tools to improve PR/AI for first and subsequent AI (Nebel et al., 2000). In spite of advances made in synchronization methods and the increased utilization of TAI protocol for first postpartum AI, greater than 60% of cows will require reinsemination. Research has shown that second and greater inseminations have much lower PR/AI than the first AI (Fricke et al., 2003; Chebel et al., 2006; Sterry et al., 2007), and therefore, there is a need to develop a resynchronization protocol that optimizes the PR/AI in lactating Holstein dairy cows.

REVIEW OF LITERATURE

Excellent dairy cattle reproductive management requires knowledge and understanding of reproductive physiology. The length of the estrous cycle in cows is 18-24 days, and includes two phases; the follicular phase and luteal phase. On average the length

of the estrous cycle is 22.9 ± 0.7 days (Sartori et al., 2004) and in high producing dairy cows, there are two follicular waves during the estrous cycle (Savio et al., 1988). The follicular phase is 4 days in length and is comprised of two stages, proestrus followed by estrus. The luteal phase is 18 days in length and is comprised of metestrus and diestrus stages. During the follicular phase, the predominant ovarian structure is the follicle, which contains the oocyte and produces 17β-estradiol (E_2). During the late follicular phase, E_2 provides a positive feedback mechanism to the surge center of the hypothalamus, causing a surge of GnRH and subsequently a pre-ovulatory surge of luteinizing hormone (LH) from the anterior pituitary, which causes ovulation. Upon ovulation, during the early luteal phase, reorganization of theca and granulosa cells into small and large luteal cells results in the formation of the corpus luteum (CL). The CL then synthesizes and secretes P_4, which provides a negative feedback mechanism to the tonic center of the hypothalamus reducing the frequency of GnRH and subsequent LH secretion. The knowledge about the aforementioned hormones and their profile during the estrous cycle, led the scientists to develop various systematic breeding protocols to take advantage of artificial insemination.

HORMONES

Gonadotropin Releasing Hormone

Gonadotropin releasing hormone is synthesized and secreted from the hypothalamus and causes the release of two gonadotropins; LH and follicle stimulating hormone (FSH) from the anterior pituitary (Senger, 2012). Gonadotropins are important for growth, development and maturation of a dominant follicle and steroid hormone (E_2/P_4) synthesis and secretion.

Gonadotropin releasing hormone is commonly used at the initiation of synchronization protocols to cause a surge of LH, which results in ovulation or luteinization of any dominant follicle and subsequently stimulating another follicular wave. If there is no dominant follicle present when GnRH is administered, ovulation or lutenization of a follicle will not occur. Gonadotropin releasing hormone is also used at the end of TAI synchronization protocols to induce ovulation before or at the time of AI. Some examples of GnRH products include Factrel® (100 μg/dose; Zoetis Inc.), Cystorelin® (100 μg/dose; Merial), and Fertagyl® (86 μg/dose; Merck Animal Health).

Progesterone

Progesterone is synthesized and secreted by small and large luteal cells of the CL during the luteal phase of the estrous cycle. Progesterone is required prior to ovulation in order to prime the uterus for embryonic development. If P_4 does not increase prior to ovulation, there is an up regulation of E_2 causing an increase in oxytocin (OXY) receptors (OTR) in the endometrium, thus resulting in premature secretion of $PGF_{2\alpha}$ and subsequent luteolysis (Inskeep, 2004). However, if P_4 is given at the proper dosage duration before the LH surge, ovulation can be prevented until P_4 concentrations decrease (Inskeep et al., 1973).

Controlled internal drug releasing (CIDR) inserts are commonly used in synchronization programs. These intravaginal inserts release P_4 (1.38 mg) into the vaginal mucous, and the amount of P_4 released into circulation is dependent on the surface area of the CIDR that comes in contact with the vaginal mucous (Rathbone et al., 2002). Progesterone from CIDR inserts suppresses estrus, making it a useful tool for estrous synchronization. Controlled internal drug releasing inserts maintain high plasma P_4 concentrations, which causes a negative feedback mechanism on the hypothalamus

preventing LH surge and ovulation (Inskeep et al., 1973; Savio et al., 1993). Upon removal of CIDR inserts, there is a rapid decline in systemic P_4 concentrations. In the absence of CL, this rapid decline in P_4, allows for an increase in gonadotophin secretion, follicular growth and increase in E_2 concentrations.

Prostaglandin

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is a lipid hormone that is synthesized and secreted by the uterine endothelium and causes the regression of the CL and therefore decrease in P_4 synthesis and secretion (Senger, 2012). In the absence of a conceptus, the initial pulses of $PGF_{2\alpha}$ stimulate the pulsatile secretion of OXY, which begins the process of CL regression known as luteolysis. Estrogen stimulates OTR in the uterus increasing uterine responsiveness to OXY (Inskeep, 2004). This event stimulates further release of $PGF_{2\alpha}$ and completes luteolysis (Silvia et al., 1991). Luteolysis is the event by which $PGF_{2\alpha}$ decreases LH receptors on luteal cells and causes necrosis and apoptosis of luteal tissue leading to a decrease in synthesis and secretion of P_4 (Senger, 2012). If administered exogenously, $PGF_{2\alpha}$ causes a decline in concentrations of systemic P_4, promoting estrus and facilitating AI administration. The administration of $PGF_{2\alpha}$ has been shown to be more effective in causing luteolysis if cows are in the diestrus phase between days 7 and 17 of the estrous cycle (Stevenson et al., 1989).

The two predominant sources of $PGF_{2\alpha}$, that are currently commercially available are Estrumate® (Merck Animal Health, 500 μg cloprostenol/dose) and Lutealyse® (Zoetis, 25 mg dinoprost/dose). These drugs are able to mimic endogenous $PGF_{2\alpha}$ and regress existing CL.

ESTROUS SYNCHRONIZATION PROGRAMS

It has been reported that heat detection (HD) efficiency in the majority of dairy herds is less than 50% (Nebel et al., 2000; Overton, 2006; Durkin, 2010). Due to inefficient heat detection, ovulation synchronization programs and timed AI (TAI) have become a valuable tool for dairy producers to improve AI submission rates (Nebel et al., 2000).

Approximately, 87% of US dairy producers use a synchronization protocol for first AI, such as Presynch (Figure 2.1) + Ovsynch (Figure 2.2), Cosynch (Figure 2.3), or Ovsynch or Cosynch without presynchronization (Pursley et al., 1997; Caraviello et al., 2006). Presynchronization consists of two $PGF_{2\alpha}$ injections 11 to 14 days apart (Figure 2.1). The initial $PGF_{2\alpha}$ injection is given to regress any existing CL thus decreasing P_4 and its inhibitory effects on gonadotropin secretion (Figure 2.1). Between the initial $PGF_{2\alpha}$ and the second $PGF_{2\alpha}$ is administered 11 to 14 days later. If cows are not anovular, greater than 90% of cows would have a CL present and the second $PGF_{2\alpha}$ would cause luteolysis and follicular turnover and therefore begin another follicular wave. Initiation of an Ovsynch (Figure 2.2) or Cosynch (Figure 2.3) protocol would begin 12 to 14 days later, therefore cows should be synchronized to day 5 to 12 of the estrous cycle when the initial GnRH is administered. Ovsynch begins with an initial GnRH to cause ovulation or luteinization of a dominant follicle and then 5 to 7 days later $PGF_{2\alpha}$ is administered to cause luteolysis of the existing and potentially newly developed CL. Forty-eight or fifty-six hours after $PGF_{2\alpha}$ is administered, cows are given a second GnRH injection. This second GnRH injection induces ovulation of the dominant follicle recruited after the first GnRH injection. Timed AI occurs 16 to 24 hours after the second GnRH injection as ovulation should occur 30 to 36 hours after the second GnRH (Pursley et al., 1997). Cosynch similar to Ovsynch with the

only difference being the second GnRH injection is given simultaneously with TAI and occurs 2 to 3 days after $PGF_{2\alpha}$, thereby reducing the number of animal handlings (Geary and Whitter, 1998). Ovsynch and Cosynch protocols can either have one or two $PGF_{2\alpha}$ injections 12 to 24 hours apart.

A recent study in dairy heifers synchronized for first AI using a 5-day CIDR-Cosynch protocol, investigated the effects of the initial GnRH as well as one or two $PGF_{2\alpha}$ injections 24 hours apart following CIDR removal on PR/AI (Lima et al., 2013). In this study, dairy heifers that received the initial GnRH and 2 injections of $PGF_{2\alpha}$ had a greater PR/AI compared to those that did not receive the initial GnRH and either one or two injections of $PGF_{2\alpha}$ (Lima et al., 2013). However, when comparing either one or two injections of $PGF_{2\alpha}$ injections without the initial GnRH, there was no difference in PR/AI despite the fact more cows in the two $PGF_{2\alpha}$ injection group exhibited complete luteolysis, which was determined by P_4 concentrations at time of AI (Lima et al., 2013). Contrary to that study, another study in dairy heifers examining the effect of a 5-day CIDR-Cosynch resynchronization protocol with the initial GnRH 28 days after first AI showed no difference in PR/AI between one or two $PGF_{2\alpha}$ injections 12 hours apart on day 5 of the experimental protocol (Rabaglino et al., 2010). Given that the Lima et al. (2013) study was examining the effects of one or two $PGF_{2\alpha}$ on the first AI and Rabaglino et al. (2010) study examined these effects on resynchronization may help to explain some of the differences in PR/AI. However, both of these studies were done in dairy heifers and it has been well established that there are differences in fertility between dairy heifers and lactating dairy cows. In lactating dairy cows presynchronized to the first AI with two $PGF_{2\alpha}$ injections 14 days apart on day 36 and 50 postpartum and then synchronized using a 5-day Cosynch protocol 11 days

later (Santos et al., 2010). In this study, the researchers investigated the effects of one or two $PGF_{2\alpha}$ injections 24 hours apart (Santos et al., 2010). There was an improvement in PR/AI when two $PGF_{2\alpha}$ injections were administered during the protocol (Santos et al., 2010). The authors of the previously mentioned studies suggested that two $PGF_{2\alpha}$ injections improved PR/AI when the initial GnRH was administered as accessory CL may have formed and two $PGF_{2\alpha}$ injections may have caused a more complete luteolysis and subsequent decrease in P_4 at the time of AI (Santos et al., 2010; Lima et al., 2013).

Figure 2.1 Presynchronization protocol. Two injections of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injections 11 to 14 days apart following before the voluntary waiting period (VWP) of 50 to 60 days postpartum. The initial $PGF_{2\alpha}$ injection is given to regress any existing corpa lutea (CL) thus decreasing progesterone (P_4) and its inhibitory effects on gonadotropin secretion. The second $PGF_{2\alpha}$ is administered 11 to 14 days later. Cows that are not anovular (> 90%) will have a CL present at the second $PGF_{2\alpha}$ injection would cause luteolysis and follicular turnover. Ovsynch or Cosynch is initiated with gonadotropin releasing hormone (GnRH) injection 12 to 14 days later in which cows should be during day 5 to 12 of the estrous cycle. During presynchronization, cows are monitored for estral behavior (heat detection; HD) and any cows that are detected in estrus may be bred and therefore not subjected to completion of Ovsynch or Cosynch protocol.

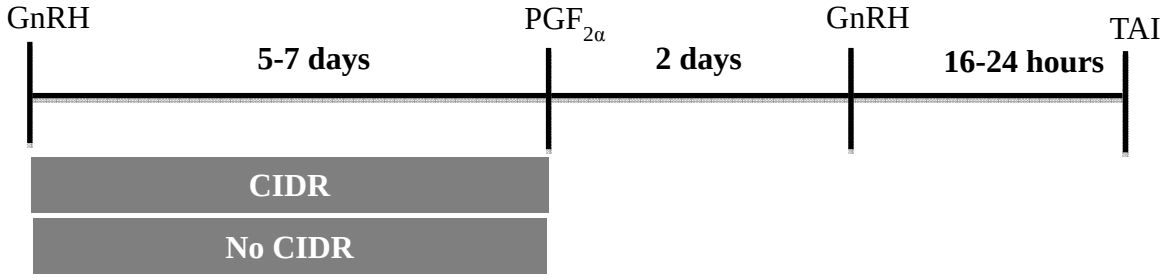

Figure 2.2 Ovsynch or Ovsynch+CIDR systematic breeding. Gonadotropin-releasing hormone (GnRH) initially administered to cause ovulation of a dominant follicle. Controlled internal drug release (CIDR) inserts may be used to increase progesterone (P_4) concentrations for 5 to 7 days (only for Ovsynch+CIDR) between initial GnRH and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) administration. On day 5 or 7 CIDRs are removed and $PGF_{2\alpha}$ is given to regress any corpus luteum (CL) that may have developed. The second GnRH is administered 2 to 3 days later to cause ovulation and timed artificial insemination (TAI) occurs 16 to 24 hours after second GnRH.

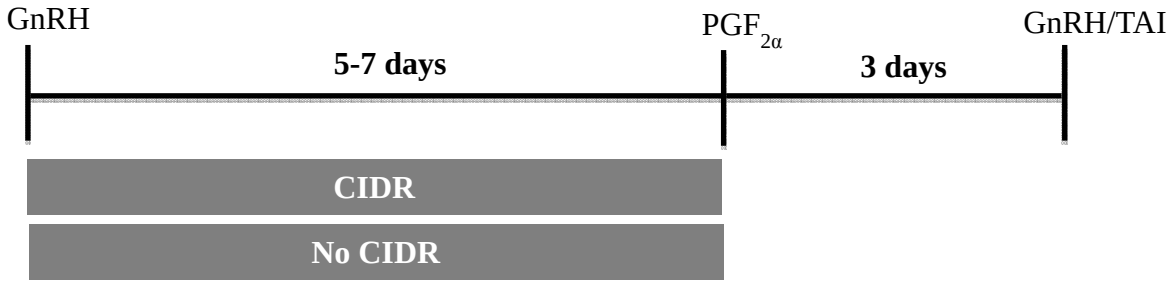

Figure 2.3 Cosynch or Cosynch+CIDR systematic breeding. Gonadotropin-releasing hormone (GnRH) is initially administered to cause ovulation of a dominant follicle. Controlled internal drug release (CIDR) inserts may be used to increase progesterone (P_4) concentrations for 5 to 7 days (only in Cosynch+CIDR) between the initial GnRH and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). On day 5 or 7 CIDRs are removed and $PGF_{2\alpha}$ is given to regress any corpus luteum (CL) that may have developed. The second GnRH is administered 3 days later and simultaneously with timed artificial insemination (TAI) to minimize animal handling.

The use of a Presynch program prior to Ovsynch or Cosynch has been shown to be very effective, as 72% of cows will regress the CL to the second $PGF_{2\alpha}$ injection 11 days

after the first $PGF_{2\alpha}$ injection of a Presynch protocol (Lucy et al., 1986). Also approximately 80% of cows will initiate a new estrous cycle if presynchronized with two $PGF_{2\alpha}$ then subjected to an Ovsynch protocol (Bello et al., 2006). Presynch-Ovsynch protocols allow for better synchrony of ovulation, which results in a greater PR/AI (Moreria et al., 2000; El-Zarkouny et al., 2004a). This increase in P/AI is due to the timing of first GnRH injection relative to the stage of the cycle when the Ovsynch protocol is initiated. Using Presynch-Ovsynch, it has been shown that cows treated with GnRH between d 5 and 9 of the estrous cycle and in the presence of a CL, results in an enhanced response to the initial GnRH with ovulation of the dominant follicle, better synchrony of ovulation at the end of the synchronization protocol, and ultimately improved PR/AI (Vasconcelos et al., 1999; Moreira et al., 2000). In fact, Vasconcelos et al. (1999) reported reduced P/AI when synchronization protocols were initiated at random stages of the estrous cycle, because 10 to 30% of cows failed to have a synchronized ovulation. Presynchronization as the name implies, is a protocol that "pre-synchronizes" cows to the early stage of the estrous cycle for optimum response to GnRH (DeJarnette, 2005). Many studies have shown that a higher PR/AI is observed when a CL is present at the final $PGF_{2\alpha}$ 72 hours prior to AI and when P_4 is high (Moreira et al., 2000; Stevenson et al., 2012b). Equally, if P_4 is low when $PGF_{2\alpha}$ is administered a lower overall PR occurs (Xu et al., 1996). This may be attributed to improper or insufficient P_4 priming of the uterus for embryo implantation and development. Also, it has been shown that synchronization using Ovsynch is most effective if initiated during the mid luteal phase of the estrous cycle (Vasconcelos et al., 1999; Stevenson et al., 2007; Green et al., 2011). The purpose of a presynchronization protocol is to synchronize cows to the mid-luteal phase of the estrous cycle at the initiation of Ovsynch or Cosynch.

Yet, studies have shown that between 6 to 59% of high producing Holstein dairy cows will not resume cyclicity and may not have a CL 60 days postpartum, when the first synchronization and AI would occur (Cerri et al., 2004; Stevenson et al., 2007; Santos et al., 2009). This may indicate that the presynchronization may not be as effective in all cows.

Progesterone Supplementation

The addition of a CIDR during Ovsynch or Cosynch following the initial GnRH helps to increase circulating P_4 and prevents ovulation of any follicle that did not respond to the initial GnRH injection. Studies have shown either no difference in PR/AI or an improvement in P/AI when CIDRs were used in an Ovsynch protocol in dairy heifers and cows (Taylor and Rajamahendran, 1991; El-Zarkouny et al., 2004a; Cerri et al., 2009a; Lima et al., 2009; Dewey et al., 2010; Rabaglino et al., 2010; Green et al., 2011). It has been suggested that any decrease in PR/AI with the use of CIDR has been attributed to the development of a persistent dominant follicle, which results in ovulation of an aged oocyte thus decreasing embryo quality and embryo development (Savio et al., 1993). However, the majority of research has indicated no difference or an increase in PR/AI with the addition of CIDR's during Ovsynch or Cosynch. For example, an improvement in PR/AI up to 5% was observed when P_4, in the form of a CIDR (CIDR+Ovsynch), was administered during the 7 d between the first GnRH and the $PGF_{2\alpha}$ injection in the Ovsynch protocol (Chebel et al., 2006). Increased PR/AI was reported when dairy cows were synchronized using P_4 containing and $PGF_{2\alpha}$ (Xu et al., 1997).

Progesterone supplementation prevents ovulation in the small percentage of cows that do not respond to the initial GnRH in an Ovsynch protocol, and also prevent premature luteolysis of a CL (Vasconcelos et al., 1999; Xu and Burton, 2000; Lima et al., 2009).

Progesterone supplementation may also help inhibiting release of gonadotropins (LH/FSH) and follicle turnover (Taylor and Rajamahendran, 1991). Using lactating dairy cows, Cerri et al. (2009a) showed that incorporation of CIDR into the Ovsynch protocol for first AI helped induce cyclicity. Also, in lactating dairy cows, a CIDR-Ovsynch resynchronization protocol beginning on day 28 following first TAI, was most effective in increasing PR/AI if cows were during the late luteal phase of the estrous cycle at initiation of the protocol (Green et al., 2011). A study in dairy heifers examining the effects of a 5-day Cosynch-TAI resynchronization protocol with and without a CIDR insert showed a 12.5% increase in PR/AI when heifers received a CIDR for 5 days between the initial GnRH and $PGF_{2\alpha}$ 28 days after the first AI (Rabaglino et al., 2010). Overall, it appears that CIDR may help induce cyclicity in lactating cows, anovular cows, and heifers, as well as tighten estrus in non-pregnant lactating cows (Chenault et al., 2003; Cerri et al., 2009a; Rabaglino et al., 2010; Green et al., 2011). Given that CIDR-Ovsynch improved pregnancy rate to first AI, it is plausible that a similar protocol may have beneficial effects when used in a resynchronization program. Previous research supported this hypothesis and showed that use of CIDR-Ovsynch as the resynchronization protocol resulted in greater P/AI compared to Ovsynch alone (El-Zarkouny and Stevenson, 2004b; Dewey et al. 2010).

5-day versus 7-day CIDR + Ovsynch

Shortening the duration of CIDR treatment from 7-days to 5-days and increasing the interval between CIDR removal and AI may enhance PR/AI. Studies have shown that there is either improvement or no difference in PR/AI when CIDR's are used for 5-days versus 7-days in an Ovsynch or Cosynch protocol in beef cows and beef heifers as well as dairy cows and dairy heifers (Bridges et al., 2008; Lopes et al., 2009; Ahmadzadeh et al., 2010; Santos

et al., 2010). Prolonged P_4 treatment may result in development of persistent follicle and an aged oocyte (Bridges et al., 2008; Lopes et al., 2009; Ahmadzadeh et al., 2010; Santos et al., 2010). Ovulation of an aged oocyte may cause a reduction in embryo quality as well as P/AI (Cerri et al., 2004; Cerri et al., 2009b). The use of a 5-day CIDR may reduce the period of prolonged follicular dominance. Presence of a younger follicle along with a longer interval between CIDR removal and AI (64 to 72 hours) helps increase the length of the proestrus period, enhancing the estrogenic capacity of the ovulatory follicle and also embryo quality and ultimately improving PR/AI (Bridges et al., 2008; Lopes et al., 2009; Ahmadzadeh et al., 2010; Santos et al., 2010). Collectively, these reasons may justify reducing the duration of CIDR treatment from 7 days to 5 days.

Research on the effect of the 5-day CIDR-Ovsynch for a synchronization or resynchronization program in lactating cows is limited. To our knowledge there is only one study investigating the use of the 5-day CIDR-Ovsynch for resynchronization in dairy cows (Bisinotto et al., 2010). These researchers reported improved PR/AI for the 5-day CIDR-Ovsynch resynchronization program (starting on d 34 post-insemination) as compared to the 5-day Ovsynch protocol alone. It should be noted that in the Bisinotto et al. (2010) study, cows received two injections of $PGF_{2\alpha}$ on day 5 and 6 (24 hours apart) of the experimental protocol.

RESYNCHRONIZATION PROTOCOLS

Successful reproductive programs for dairy herds should encompass three essential steps: (1) submission of all cows for first postpartum AI shortly after the end of the VWP, (2) early identification of non-pregnant cows after each AI and (3) resynchronization and

resubmission of cows to a second or greater AI service that fail to conceive to the previous AI (Fricke et al., 2003).

Some studies indicate that 20 to 40% of cows can be anovular at the beginning of the first postpartum synchronization (60 DIM), which accounts for a portion of the 6 to 59% of cows that will not begin cyclicity by first synchronization (Moreira et al., 2000; Cerri et al., 2004; Santos et al., 2004; Stevenson et al., 2007; Santos et al., 2009). Others also reported that only 40% of high producing lactating Holstein dairy cows will actually conceive to the first postpartum AI (Pursley et al., 1997; Jobst et al., 2000; Fricke et al., 2003; Cerri et al., 2004; Chebel et al., 2006; Galvão et al., 2007). Therefore, approximately 60% of lactating dairy cows fail to conceive to the first AI and must be subjected to a second synchronization and AI.

In order to improve PR, reduce DO, and enhance production efficiency, it is essential to develop and implement an effective resynchronization strategy for subsequent AI services in cows that have not conceived to a previous AI. The overall goal of a successful resynchronization program is to minimize the interval between the first and subsequent inseminations by determining non-pregnant cows as quickly as possible, reinseminating them so they become pregnant sooner. However, PR for the second and third AI's is much lower than the first AI (Fricke et al., 2003; Chebel et al., 2006; Sterry et al., 2007). Bilby and co-workers (2012) showed that < 30% of cows resynchronized with Ovsynch will actually conceive, and in fact, second insemination PR are reported to be 5 to 10% points lower than first inseminations (Lucy, 2012).

Given that detection of estrus remains a challenge after first AI on many farms, as well as a large proportion of dairy cows requiring more than one insemination, coupled with

the fact that PR/AI to second and greater TAI is less than first AI (Fricke et al., 2003; Chebel et al., 2006; Sterry et al., 2007), there is a need to develop and optimize a resynchronization program that can result in greater fertility compared with existing TAI resynchronization protocols. The majority of cows do not return to estrus 21 days following first AI if they do not conceive, because in reality, the return to estrus can be between 18 and 28 days following first AI (Chebel et al., 2006). Many factors can affect the length of return to estrus following AI, including early embryonic loss or failure of synchronization to first synchronization. Synchronization failure of first AI can be caused by a lack of compliance to hormone administration or other physiological reasons such as unresponsiveness of CL at the time of $PGF_{2\alpha}$ and/or existence of a small dominant follicle, which does not ovulate to the final GnRH before AI in TAI protocols such as Ovsynch (Bartolome et al., 2005). Another reason could be early regression of the CL due to premature secretion of $PGF_{2\alpha}$, which may cause early embryonic loss delaying the resumption of cyclicity after the AI (Bartolome et al., 2005). Therefore, there is a larger variation for days in return to estrus following AI. Because of low HD efficiency (< 50%) and wider window of time in return to estrus post-AI, many open cows are not identified and not resynchronized until pregnancy diagnosis, 32 to 35 days following first AI, when cows are in various stage of anew estrus cycle following the previous AI.

Timing of Resynchronization Relative to Previous AI

Various times for initiation of resynchronization following first AI have been studied. Resynchronization has been shown to improve PR/AI when initiated 32 days following first insemination. This time interval is chosen based on the average the length of the estrous cycle (21 days). If cows do not become pregnant to AI, theoretically, they should

exhibit estrus 21 days after insemination. In fact, 10% of cows will return to estrus by day 22 post first AI (Dewey et al., 2010). Therefore, if Ovsynch resynchronization begins 27 to 32 days following first AI, cows will be synchronized during day 5 to 10 of the new estrous cycle, which may improve PR/AI as the first GnRH in the Ovsynch protocol will occur when there is greater chance for a dominant follicle to be present (Vasconcelos et al., 1999; Moreira et al., 2000; Dewey et al., 2010). However, from a management standpoint, pregnancy diagnosis usually occurs once a week; therefore, cows may be determined open between 32 and 39 days post first AI placing them between day 10 and 17 of the estrous cycle which may not be an optimal time to initiate the Resynch-Ovsynch protocol.

By using ultrasonography Bartolome and co-workers (2005) examined the follicular dynamics on day 30 after first AI in lactating dairy cows. The results of this study indicated that the 45% of cows were during the diestrus stage of the estrous cycle, which if they were open would respond to the initial GnRH in an Ovsynch or Cosynch resynchronization protocol (Bartolome et al., 2005). The remaining 55% of cows were either during the metestrus stage, proestrus stage with ovarian cysts, or were anestrus (Bartolome et al., 2005). Normally these groups of cows do not respond to the initial GnRH in an Ovsynch or Cosynch resynchronization protocol, and therefore would not be synchronized and become pregnant to subsequent AI. Of the 55% of open cows, authors suggested four possible reasons for failure of conception following first AI (Bartolome et al., 2005). The four reasons were abnormal estrous cycles (maybe due to failure of previous synchronization), early regression of the CL, embryo loss with a delayed return to estrus, or failure of ovulation (Bartolome et al., 2005). Also, it was indicated that cows during metestrus 30

days after first AI have already initiated another follicular wave, and therefore would lack a dominant follicle and be unresponsive to the initial GnRH in an Ovsynch protocol.

Collectively, the previously mentioned studies indicate that 16 to 28% of cows lack a CL 32 to 34 days after the first TAI (Fricke et al., 2003; Bartolome et al., 2005; Sterry et al., 2007; Bartolome et al., 2009; Bisinotto et al., 2010). Therefore, at the initiation of resynchronization, some cows will not be in the mid-luteal phase of the estrous cycle, which may not be an optimal scenario initiation of an Ovsynch protocol as response to the initial GnRH would be more likely (Vasconcelos et al., 1999). In addition cows may not have a dominant follicle to respond to the first GnRH injection with ovulation and/or luteinization of follicles. In fact, Bisinotto et al. (2010) showed that ovulation incidence to first GnRH in dairy cows subjected to a Resynch-Ovsynch or 5d CIDR-Ovsynch protocol was only 35% overall. If cows fail to ovulate to the first GnRH injection, the follicle induced to ovulate at the end of the synchronization protocol may have undergone prolonged dominance, which leads to the ovulation of an older oocyte, and consequently, reduced embryo quality (Cerri et al., 2009) and decreased P/AI (Chebel et al., 2006).

Initiation of a Resynch-Ovsynch one week prior to pregnancy diagnosis and $PGF_{2\alpha}$ at the time of open diagnosis has shown no detrimental effect on pregnant cows, but this method can significantly increase synchronization costs as all cows receive GnRH (Lucy, 2012). A study by Chebel et al. (2003) indicated that Ovsynch initiated on day 21 after first AI resulted in a higher PR while Fricke et al. (2003) showed an increase in PR on day 26 (34%) or day 33 (38%) rather than day 19 (23%). Initiation of an Resynch-Ovsynch protocol 21 days following the first AI results in 72% of cows with > 2.35 ng/mL P_4 at the initiation of synchronization, and only 33.4% were actually pregnant (Chebel et al., 2003). The

evidence showed that Resynch-Ovsynch with a CIDR initiated 32 days after first AI and in the presence of a CL, resulted in greater P/AI and decrease in pregnancy loss compared to Resynch-Ovsynch and no CIDR (Chebel et al., 2006; Bisinotto et al., 2010). A study by Green et al (2011) observed comparable results when lactating Holstein cows were resynchronized 28 or 35 days following first AI. Overall, dairy cows subjected to resynchronization on average 32 days after the first AI will be at various stages of the estrous cycle. This variation may reduce the cows' responses to resynchronization protocols and negatively affect PR/AI.

Methods of Resynchronization

The original method for resynchronizing dairy cows was a single injection of $PGF_{2\alpha}$ at the time of open diagnosis. If a CL is not fully functional or is absent at the time of pregnancy diagnosis, $PGF_{2\alpha}$ will not elicit an effect (Lucy, 2012). If $PGF_{2\alpha}$ is given to a pregnant cow, abortion will result, which will extend DO and increase the cost to the producer. Because of the above issues, this method is not very common.

Another method used for resynchronization, is a Double-Ovsynch protocol. This protocol requires two Ovsynch programs seven days apart upon open diagnosis. Some studies have shown an increase in PR/AI for the use of Double-Ovsynch compared to a single Ovsynch following open diagnosis. For example, a study done by Giordano et al. (2012) showed an increase in PR/AI using a Double-Ovsynch protocol compared to one Ovsynch when the protocols were initiated 32 days following first TAI. However, this protocol requires more animal handlings, doubles the costs for resynchronization and increases the amount of time for synchronization. On average, it costs $12.90 per cow for an Ovsynch protocol and this includes hormones, semen and labor costs associated with

synchronization (Stevenson, 2012). Currently, one of the most common methods for resynchronizing lactating dairy cows is a single Ovsynch protocol. Approximately 77% of dairy producers use some sort of synchronization protocol for resynchronization and 69% of them use a single Ovsynch protocol (Caraviello et al., 2006).

Ovsynch/Cosynch + CIDR

A study by DeJarnette, et al (2001) indicated that 20% of cows will ovulate a dominant follicle between the first and second GnRH injections when an estrous synchronization protocol is initiated at random stages of the estrous cycle. Therefore, the use of a CIDR to prevent ovulation or premature luteolysis may prevent these circumstances and result in a more successful synchrony especially during resynchronization. In lactating Holstein dairy cows, a resynchronization protocol, using a 7-day CIDR-Ovsynch program, 18 days following the first TAI, was shown to be an effective method to synchronize cattle to the diestrus phase of the estrous cycle (Green et al., 2011). The authors made this conclusion as nearly all cows exhibited > 1 ng/mL P_4 at the initial GnRH (Green et al., 2011). Still, studies using CIDRs for resynchronization programs have shown inconsistant results. Chenault et al. (2003) showed no beneficial effects in lactating Holstein cows previously inseminated and then presynchronized with $PGF_{2\alpha}$ and the use of 7-day CIDR 14 days later (AI was administered based on detection of estrus). The authors hypothesized that the observed decrease in PR with the use of CIDRs was due to an increase in mucosal scores when CIDRs were used, which suggested severe vaginitis. Others (Dewey et al., 2010) have shown that the addition of CIDRs in Ovsynch protocol increased PR/AI when lactating cows were presynchronized with GnRH 7 days before initiation of a 7-day CIDR-Ovsynch protocol.

5-day versus 7-day CIDR

Recent research in lactating dairy cows, beef cows, and beef and dairy heifers (Bridges et al., 2008; Lopes et al., 2009; Ahmadzadeh et al., 2010; Rabaglino et al., 2010; Santos et al., 2010) investigated the effect of shortening the interval (from 7 to 5 d) between the initial GnRH and the injection of $PGF_{2\alpha,}$ in the presence of CIDR, and a longer interval between CIDR removal in AI. In fact, in dairy heifers resynchronized 28 days after first TAI, a 5-day CIDR-Cosynch protocol helped to increase PR/AI by 12.5% compared to a 5-day Cosynch protocol (Rabaglino et al., 2010).

Some of the previous studies (Bridges et al., 2008; Lopes et al., 2009; Ahmadzadeh et al., 2010) investigated the effects of a 5-day CIDR versus a 7-day CIDR for first AI. Collectively, these studies have shown either an improvement or no difference in PR/AI when CIDR's were used for 5 versus 7 days in an Ovsynch or Cosynch protocol. The purpose of reducing the interval of CIDR inserts is to prevent prolonged P_4 treatment, which may result in ovulation of an aged oocyte, especially if ovulation to the initial GnRH does not occur (Bridges et al., 2008; Lopes et al., 2009; Santos et al., 2010). Ovulation of an aged oocyte has been shown to reduce embryo quality as well as decrease PR/AI (Cerri et al., 2004; Cerri et al., 2009b). Therefore, by using a 5-day CIDR, there will be a reduction of prolonged follicular dominance and increased length of proestrus following CIDR removal (because AI is commenced 64 to 72 h instead of 48 h after CIDR removal), which may ultimately enhance the estrogenic capacity of the ovulatory follicle and subsequent P_4 production by the new CL. This in turn may aid in embryo implantation and quality (Bridges et al., 2008; Lopes et al., 2009; Santos et al., 2010).

Collectively, these results support the potential use of a 5-day CIDR versus a 7-day CIDR; however, there is currently a limited amount of research on the use of CIDR inserts for resynchronization in lactating dairy cows. To our knowledge, there is only one study that examined the effects of a 5-day CIDR-Ovsynch resynchronization protocol in lactating dairy cows. In that study (Bisinotto et al., 2010) resynchronization began 34 days post first insemination, and the authors showed an improvement in PR/AI when lactating dairy cows were resynchronized with a 5-day CIDR-Ovsynch protocol compared to a 5-day Ovsynch protocol. However in that study, cows received two $PGF_{2\alpha}$ injections at the time of CIDR removal (Bisinotto et al., 2010). Consequently, research investigating the use of a 5-day CIDR-Cosynch in a resynchronization protocol is warranted.

Effects of Initial GnRH in Ovsynch Protocols

Given that the majority of cows 30 days after first AI will not be during the optimal stage of the estrous cycle for the initiation of resynchronization protocol, the induction of ovulation or luteinization of the dominant follicle by GnRH (as done in protocols such as Ovsynch or Cosynch), therefore would not be effective or even necessary (Bartolome et al., 2005). Studies have shown that 18 to 27% of cows lack a CL on day 32 to 42 following first AI (Fricke et al., 2003; Sterry et al., 2007; Bartolome et al., 2009; Bisinotto et al., 2010). In addition, another study indicated that only 35% of cows ovulated to the initial GnRH in a Resynch-Ovsynch protocol (Bisinotto et al., 2010).

The purpose of the initial GnRH injection in an Ovsynch protocol is to initiate a new follicular wave. This new wave occurs on average 1.6 to 2.5 days following GnRH administration (Pursley et al., 1995; Bello, et al., 2006). However, if the Ovsynch protocol is initiated at random stages of the estrous cycle, approximately 10 to 30% will fail to

synchronize to ovulation and have a decreased PR/AI (Vasconcelos et al., 1999). In contrast, another study indicated that when Ovsynch is initiated at random stages of the estrous cycle approximately 90% of lactating dairy cows responded to the first GnRH injection, whereas only about 50% of heifers will respond (Pursley et al., 1995). However, this study refers to the first AI after cows were presynchronized using two $PGF_{2\alpha}$ 14 days apart, therefore tightening synchronization of cows.

By using a Presynch-Ovsynch protocol, cows are set up so that the initial GnRH injection is administered on day 5 to 9 of the estrous cycle and in the presence of a CL. This method increases the chance of ovulation of a dominant follicle to the initial GnRH and may improve PR/AI (Vasconcelos et al., 1999; Moreira et al., 2000). However, if GnRH is given between day 10 and 16 of the estrous cycle approximately 50% of cows will fail to ovulate to the initial GnRH injection (Vasconcelos et al., 1999). If dairy cows fail to ovulate after the initial GnRH injection in an Ovsynch protocol, there may be prolonged dominance of an ovulatory follicle resulting in ovulation of an aged oocyte (Wishart et al., 1977; Savio et al., 1993; Stock et al., 1993; Austin et al., 1999). Ovulation of an aged oocyte has been shown to cause a decrease in embryo quality as well as reduce PR/AI (Cerri et al., 2009a & 2009b). Therefore, the success of the Ovsynch protocol hinges on the stage of the estrous cycle, ovarian status at the time initiation, and ovulation response to the initial GnRH.

Use of GnRH in the CIDR+Ovsynch Timed AI Protocol

Previously published 5-day and 7-day CIDR-Ovsynch protocols recommend an injection of GnRH at the time of CIDR insertion. Nevertheless, it remains unclear whether the use of GnRH at the time of CIDR insertion in the CIDR-Ovsynch program actually

improves PR. The potential benefits of the initial GnRH injection in CIDR-based TAI protocols have been tested in both cows and heifers.

Many studies have shown no advantage of the initial GnRH in a CIDR-Ovsynch protocol to first AI. A study done by Larson et al (2006) on suckling beef cows indicated no difference in PR/AI between initial GnRH versus no GnRH in CIDR-Cosynch protocol. In addition, when both beef and dairy heifers subjected to a 5 day CIDR-Cosynch TAI, initial GnRH injection showed no improvement in PR/AI indicating that the initial GnRH may not be necessary and could potentially save producers $2.50 to $3.50 per head (Howard et al., 2009; Howard et al., 2012; Stevenson, 2012). Similarly, when dairy heifers were subjected to a 5-day CIDR-Cosynch protocol, the initial GnRH injection had no effect on overall fertility because there was a low rate of ovulation to the initial GnRH administration (Lima et al., 2011). These authors suggested that in a 5-day TAI protocol, elimination of the initial GnRH and use of a single $PGF_{2\alpha}$ injection 72 hours before the final GnRH administration will result in similar PR/AI compared to heifers that received the initial GnRH (Lima et al., 2011). However, in a more recent study, using a 5-day CIDR-Cosynch protocol, Lima et al. (2013) demonstrated that for the first AI, with and without the initial GnRH two injections of $PGF_{2\alpha}$, at CIDR removal resulted in greater PR/AI than a single injection of $PGF_{2\alpha}$ at CIDR removal. The authors suggested that the greater observed PR/AI may be due to a more complete luteolysis upon CIDR removal in the two $PGF_{2\alpha}$ injection group. However, it should be noted that these studies were conducted in dairy heifers for first AI.

In contrast, in a multiple location study in beef heifers, elimination of the initial GnRH in a CIDR-Ovsynch TAI protocol and one $PGF_{2\alpha}$ had no adverse effect on PR/AI (Lamb et al., 2006). However, he authors noted that there was significant variation among

locations and suggested that the initial GnRH injection may help to achieve consistent PR/AI across multiple locations (Lamb et al., 2006). Given the previously mentioned research, there is still a lack of evidence on the effects of the initial administration of GnRH in a CIDR-Ovsynch or Cosynch resynchronization protocol in lactating dairy cows.

OBJECTIVES & RATIONALES

HYPOTHESIS

Due to inefficient estrous detection (< 50%) efficiency across many dairy herds, ovulation synchronization programs and TAI have become a valuable tool for dairy producers to improve AI submission rates of first AI services. Given that detection of estrus remains a challenge after first AI on many dairy farms, coupled with the fact that PR/AI to second and greater TAI are less than first AI (Fricke et al., 2003; Chebel et al., 2006; Sterry et al., 2007), there is a great need to develop and optimize a resynchronization program that results in greater fertility compared with existing TAI resynchronization protocols.

Currently, resynchronizing lactating Holstein dairy cows with an Ovsynch protocol has yielded less desirable results in PR/AI compared with PR/AI when Ovsynch is used for the first postpartum AI. This is mainly due to the dynamics of the ovarian structures at the initiation of a resynchronization program, which usually occurs approximately 32 to 40 days following first service. The use of a CIDR during synchronization has been shown to tighten synchrony and prevent ovulation of a dominant follicle. Moreover, studies have shown that a shorter duration of CIDR treatment in a 5-day CIDR-Cosynch with TAI (GnRH [d 0], CIDR removal + $PGF_{2\alpha}$ [d 5], GnRH+AI [d 8]) is a viable option for breeding cows without having any detrimental effects on PR/AI. Although research has shown that

the initial GnRH injection may not be necessary in heifers, it is unclear whether the use of GnRH at the time of CIDR insertion in a CIDR-Cosynch protocol improves PR/AI in dairy cows, especially for resynchronization. The initial GnRH injection may not be necessary as it is only effective if a dominant follicle is present at initiation of the protocol. Providing that 18 to 27% of cows lack CL at 32 to 34 days after previous AI (Fricke et al., 2003; Sterry et al., 2007; Bartolome et al., 2009; Bisinotto et al., 2010) and potentially 50% of cows will fail to ovulate to the initial GnRH injection (Vasconcelos et al., 1999), resynchronized cows may not respond to the initial GnRH injection with ovulation and/or lutenization of follicles (Bisinotto et al., 2010), therefore the use of the initial GnRH may not be necessary. Therefore, the hypothesis of this study was by using a 5-day CIDR-Cosynch for resynchronization of the second AI without the initial GnRH may either improve or provide similar PR/AI, and therefore may not be necessary.

OBJECTIVES

The objectives of this study, was to determine the effect of the initial GnRH injection in a 5-d CIDR-Cosynch resynchronization protocol on PR to second AI in lactating Holstein dairy cows.

The specific objectives were to:

1.) Compare the PR to second AI in cows treated with initial GnRH (control) vs. no GnRH.
2.) Examine the effects of serum P_4 concentrations at the initiation of experiment (day 0) on PR to second AI.
3.) Compare overall reproductive performance between GnRH and no GnRH groups.
4.) Compare PR/AI in cows inseminated based on detected estrus or TAI.

MATERIALS & METHODS

ANIMALS

All animal handling procedures and treatment protocols used in this experiment were in the accordance with the protocols of the Animal Care and Use Committee at the University of Idaho (Appendix 5). This project was conducted from January to July 2014 at 5-D Dairy in Pasco, Washington. There were approximately 8,000 milking cows (Holstein and Jersey breeds), with an average 150 corrected milk (150CM) production of 35 kg/cow/day. All cows were presynchronized to first AI with two injections of $PGF_{2\alpha}$ with an average of 64 DIM to first breeding (DIMFB) and voluntary waiting period (VWP) of 50 days. The herd average DO was 114, with averages services per conception (S/C) of 2.8. The average CR to first and second AI were 38% with an overall average of 35% CR. The average pregnancy rate for December 2012 to 2013 was 23.8% and from December 2013 to June 2014 was 25.8%. For better comparison of the herd average and experimental cows for this project, 150CM, DO and TBRD greater than 104 DIM were assessed. The average 150CM was 37.7 kg/cow/day, DO was 142 days and S/C was 3. All animals were housed in free-stall barns and milked twice daily. Cows were fed a total mixed ration (TMR) twice daily, which was pushed up 4 to 6 times a day, cows also had ad libitum access to water.

CHARACTERISTICS OF RESEARCH COWS

At the initiation of the study, 476 lactating Holstein cows during 21 visits eligible for second AI, were subjected to either 5-day CIDR-Cosynch resynchronization and given the initial GnRH (control) or not given the initial GnRH (treatment). After initial analysis of the results, 429 primiparous and multiparious lactating dairy cows were used for this experiment. Of those eliminated from the study, 17 were sold prior to pregnancy diagnosis,

and 26 were bred at CIDR removal or more than 3 days after CIDR removal. Four cows were eliminated because they were re-inseminated before pregnancy diagnosis.

EXPERIMENTAL PROCEDURES

First AI was accomplished according to the management practice of the collaborating dairy. An average of 37 days following first AI, cows diagnosed non-pregnant by rectal palpation were enrolled into this experiment. During 21 weekly veterinarian pregnancy diagnoses, 476 open primiparous and multiparous Holstein cows that were eligible for a second insemination were selected for the experiment. Upon open diagnosis, 476 lactating dairy cows were randomly assigned to one of the two treatments, of which 47 were eliminated for various reasons (please refer to previous section "Characteristics of Research Cows"). Blood samples were collected at the initiation of the experiment from a subgroup of cows and analyzed for P_4 concentrations.

Treatment

On day 0 of the experiment (the day of pregnancy diagnosis), cows were randomly assigned to either treatment (no GnRH injection) or control (GnRH injection; 100μg, i.m.; Factrel®; Fort Dodge Animal Health, Fort Dodge, IA) groups. Simultaneously, a CIDR (1.38g P_4; Eazi-Breed CIDR®, Zoietis, Florham Park, NJ) was inserted for 5 days in all cows. On day 5, CIDR's were removed and $PGF_{2\alpha}$ (500 μg, i.m.; Estrumate®; Merck Animal Health Intervet Inc. Summit, NJ) was administered to all cows. Once daily, cows were monitored for estrual behavior using tail chalk from day 5 to 8 of the experiment. Cows that were detected in estrus were bred and cows that did not exhibit estrus prior to day 8 received GnRH (100 μg) and were subjected to TAI from one of three technicians. Cows that were bred based on HD were not given the final GnRH. All injections were

administered using 18 gauge 1 ½" needles and given intramuscularly (i.m.) into the gluteus medius muscle between the hooks and pins of the rump area.

Figure 2.4 Control group (n = 226) of lactating Holstein cows eligible for second artificial insemination (AI). Cows were enrolled in a 5-day CIDR-Cosynch resynchronization protocol on average 37 days after first AI. Cows that were detected in estrus (HD; day 6 or 7), received AI and were not administered gonadotropin-releasing hormone (GnRH). The remaining cows that did not exhibit estrus recieved timed artificial insemination (TAI; day 8) and were administered GnRH simultaneously.

Figure 2.5 Treatment group (n = 203) of lactating Holstein cows eligible for second artificial insemination (AI). Cows were enrolled in a 5-day CIDR-Cosynch resynchronization protocol on average 37 days after first AI. Cows that were detected in estrus (HD; day 6 or 7), received AI and were not administered gonadotropin-releasing hormone (GnRH). The remaining cows received timed artificial insemination (TAI; day 8) and were administered GnRH simultaneously.

Blood Samples and Progesterone Quantification

All blood samples were collected via coccygeal venipuncture, at the proximal ventral surface of the tail. The tail was punctured using an 18 gauge 1 ½" single use blood

collection needle. Blood samples were collected using 10 mL serum Covidien, Monoject Vacutainers® (Covidien LLC, Mansfield, MA). Within 2 hours all samples were placed on ice and transported to the University of Idaho laboratory and stored at 4º C for 18 to 24 hours. Samples were centrifuged the following day for 20 minutes at 2100 x g at 4º C. Serum was harvested and samples were stored in a freezer at -20º C until assayed for P_4 concentrations. Progesterone concentrations were quantified using a single antibody solid-phase radioimmunoassay (RIA; Siemens Health Care Diagnostic, Los Angeles, CA). The standard curve ranged from 0.1 to 40 ng/mL and the standard curve and all samples were run in duplicates. The intraassay coefficient of variance (CV) was 7.8%.

STATISTICAL ANALYSIS

Analysis of variance procedures were used to compare 150 day corrected milk production (150CM), days in milk at first breeding (DIMFB), days in milk at second breeding (DIMSB), days open (DO), parity, number of times bred before conception (TBRD), and P_4 concentration at initiation of experiment (day 0) between the two treatment groups (Appendix 4.3). These response variables were analyzed for effects on treatment, occurrence of disease within the first 60 days in milk (DIM) as well as the interactions between treatment and disease. Diseases were categorized as yes or no and included mastitis (n = 15), milk fever (n = 9), retained placenta (n = 8), metritis (n = 31), pneumonia (n = 5), displaced abomasum (n = 1), injury (n = 1), lameness (n = 104) and off feed (n = 2) (Appendix 3.1).

Logistic regression was used to analyze the main effects of treatment (GnRH versus no GnRH), day of AI (Early = day 6 or 7, Late = day 8 of experimental protocol), technician (3 technicians), sire (10 sires), and parity (primiparous versus multiparous) on overall PR/AI

(Appendix 4.1). Pregnancy rate per AI was determined by examining the odds ratio, which explains the proportion of cows that conceived versus the proportion of cows that failed to conceive. Logistic regression was also employed to analyze a subpopulation of 184 cows P_4 concentrations at initiation of the experiment (day 0). Progesterone concentrations (n =184) were categorized as low (< 1 ng/mL) or high (≥ 1 ng/mL) and analyzed for effects on overall PR/AI using the same model (Appendix 4.2).

RESULTS

RESPONSE VARIABLES

There were no differences in mean 150CM (μ = 41 kg/day), DIMFB (μ = 66 days), DIMSB (μ = 111 days) and parity (μ = 2.5 lactations) between no GnRH (treatment) and GnRH (control) groups ($P > 0.05$), indicating a similar population of animals in each group (Table 2.1). There were no effects of disease on 150CM, DIMFB, DIMSB, DO and TBRD, however the occurrence of diseases within the first 60 days postpartum were significantly greater for older cows than younger cows ($P < 0.05$) (Appendix 2.1.1). Mean serum P_4 concentrations (n = 184) on day 0 of experimental protocol did not differ ($P > 0.05$) between GnRH (n = 95) and no GnRH (n = 89) groups (3.96 ± 0.34 ng/mL treatment vs. 4.51 ± 0.35 ng/mL control; Table 2.1; Appendix 4.3). The number of cows with $P_4 \leq 1$ ng/mL, which was considered to have a non-functional CL, was similar between treatment groups.

Table 2.1 Mean ± SE for 150 day corrected milk (150CM), days in milk at first breeding (DIMFB), days in milk at second breeding (DIMSB), parity (primiparious and multiparous), and progesterone (P_4)***** concentrations between GnRH$^{\infty}$ (control; n = 226) and no GnRH$^{\infty}$ (treatment; n = 203) groups in lactating Holstein cows.

Response Variable	GnRH	No GnRH
150CM (kg)	41.38 ± 0.71	40.94 ± 0.72
DIMFB (days)	66.10 ± 1.12	66.20 ± 1.15
DIMSB (days)	111.66 ± 1.16	111.39 ± 1.19
Parity (lactation #)	2.61 ± 0.09	2.67 ± 0.09
Progesterone (ng/mL)	3.96 ± 0.34	4.51 ± 0.35

$^{\infty}$ Day 0 cows were administered gonadotropin-releasing hormone (GnRH) (control) or no GnRH (treatment) and controlled internal drug release (CIDR) inserts were administered to all cows. On day 5 CIDR's were removed and all cows received a prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection. Cows were bred based on heat detection (early; day 6 or 7) and not given the final GnRH or bred timed AI (late; day 8) and given the final GnRH at the time of insemination.

* Mean P_4 concentrations are from a subgroup of cows (n = 184).

PREGNANCY RATES PER ARTIFICAL INSEMINATION

The PR/AI did not differ (P > 0.05) between no GnRH (treatment) and GnRH (control) groups (27.09 % treatment vs. 21.23 % control; Figure 2.6). Pregnancy per AI for cows bred based on estrus (early; day 6 or 7; AI based on estrus detection; n = 50) or TAI (late; day 8, AI 3 days after CIDR removal; n = 379) did not differ (28% versus 23%; P > 0.05; Figure 2.6). There was no effect of parity by treatment interaction on PR/AI; however parity had a significant effect (P < 0.05) on overall PR/AI with first lactation (n = 119) having a greater PR/AI than lactation 2 and greater (n = 310) (31% vs. 21%; Figure 2.6). There was no effect of sire or sire by treatment interaction on PR/AI. Moreover, there was

no effect of technician by treatment interaction on PR/AI; however, there was a tendency for difference in PR/AI among tecnicians (data not shown; Appendix 4.1). Although treatment had no effect on PR/AI, there was a significant difference on DO and TBRD between no GnRH and GnRH groups, the no GnRH group had lower DO compared to the GnRH group (158.30 ± 3.51 for no GnRH versus 171.71 ± 3.43 for GnRH; $P < 0.05$). Similarly, TBRD were smaller for no GnRH compared to GnRH (3.39 ± 0.09 for no GnRH versus 3.68 ± 0.09 for GnRH; $P < 0.05$) (Table 2.2).

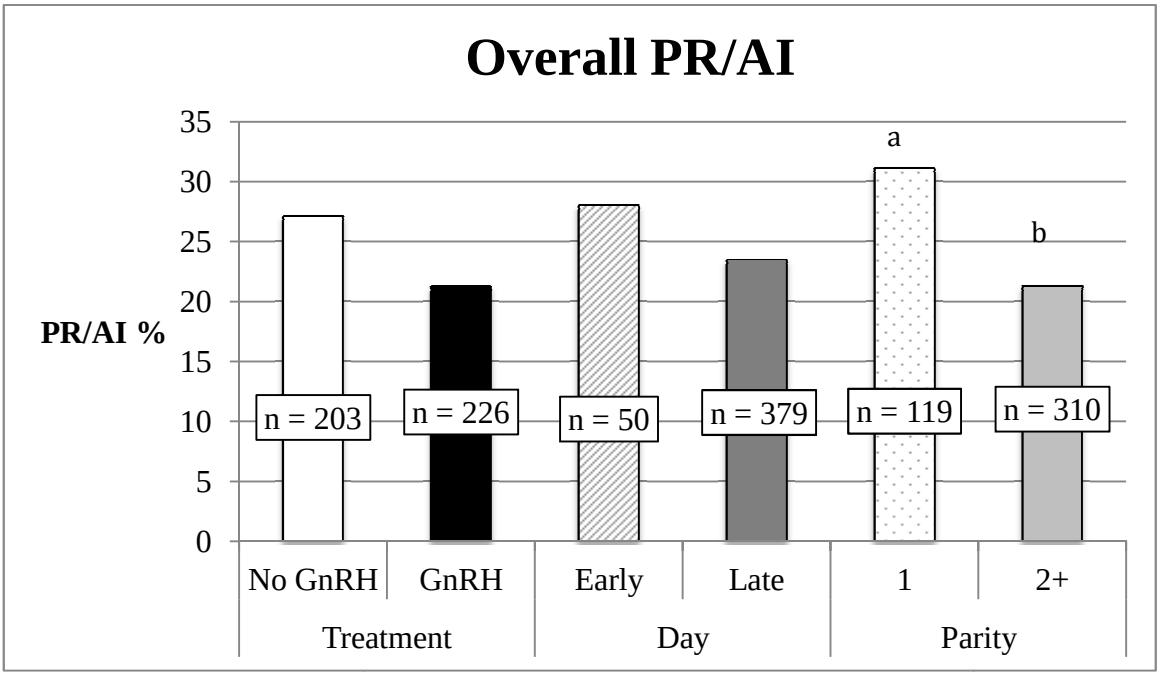

Figure 2.6 Pregnancy rates per artificial insemination (PR/AI) for initial GnRH[∞] and no-GnRH[∞] treatment, early or late bred, and primiparous and multiparous lactating Holstein cows. GnRH treatment cows received an injection of GnRH (100 ug) at the initiation of the breeding protocol. No GnRH treatment cows did not receive an injection of GnRH at the initiation of the breeding protocol. Day of artificial insemination (AI) was considered early (bred based on heat detection (HD), day 6 or 7 of experimental protocol) or late (timed AI (TAI) on day 8 of experimental protocol, and parity (primiparous vs. multiparous).

[∞] Day 0 cows were administered gonadotropin-releasing hormone (GnRH) (control) or no GnRH (treatment) and controlled internal drug release (CIDR) inserts were administered to all cows. On day 5 CIDR's were removed and all cows received a prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection. Cows were bred based on heat detection (early; day 6 or 7) and not given the final GnRH or bred timed AI (late; day 8) and given the final GnRH at the time of insemination.

[ab] Bars with different letters differ significantly ($P < 0.05$).

Table 2.2 Mean ± SE for days open (DO) and number of times bred (TBRD) for GnRH[∞] (control; n = 226) and no GnRH[∞] (treatment; n = 203) groups of both primiparous and multiparous lactating Holstein cows.

Parameter	GnRH	No GnRH
DO (days)	171.71 ± 3.43[a]	158.30 ± 3.51[b]
TBRD	3.68 ± 0.09[a]	3.39 ± 0.09[b]

[∞] Day 0 cows were administered gonadotropin-releasing hormone (GnRH) (control) or no GnRH (treatment) and controlled internal drug release (CIDR) inserts were administered to all cows. On day 5 CIDR's were removed and all cows received a prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection. Cows were bred based on heat detection (early; day 6 or 7) and not given the final GnRH or bred timed AI (late; day 8) and given the final GnRH at the time of insemination.

[ab] Means with different letter within the row differ significantly ($P \leq 0.05$).

PREGNANCY RATES & SERUM PROGESTERONE

Blood samples were collected from a subpopulation of cows (n = 184) on day 0 of the experimental protocol for P_4 quantification. Cows were classified as having either high ($\geq$ 1 ng/mL) or low (< 1 ng/mL) P_4. In this group of cows, there were no observed effects of treatment or treatment by serum P_4 categories (high versus low) on overall PR/AI. However, regardless of treatment, the odds of conception compared to failure to conceive (PR/AI) tended ($P = 0.09$) to be greater for high P_4 (n = 136) than low P_4 (n = 48) (26% for high vs. 16% for low; Figure 2.7; Appendix 4.2).

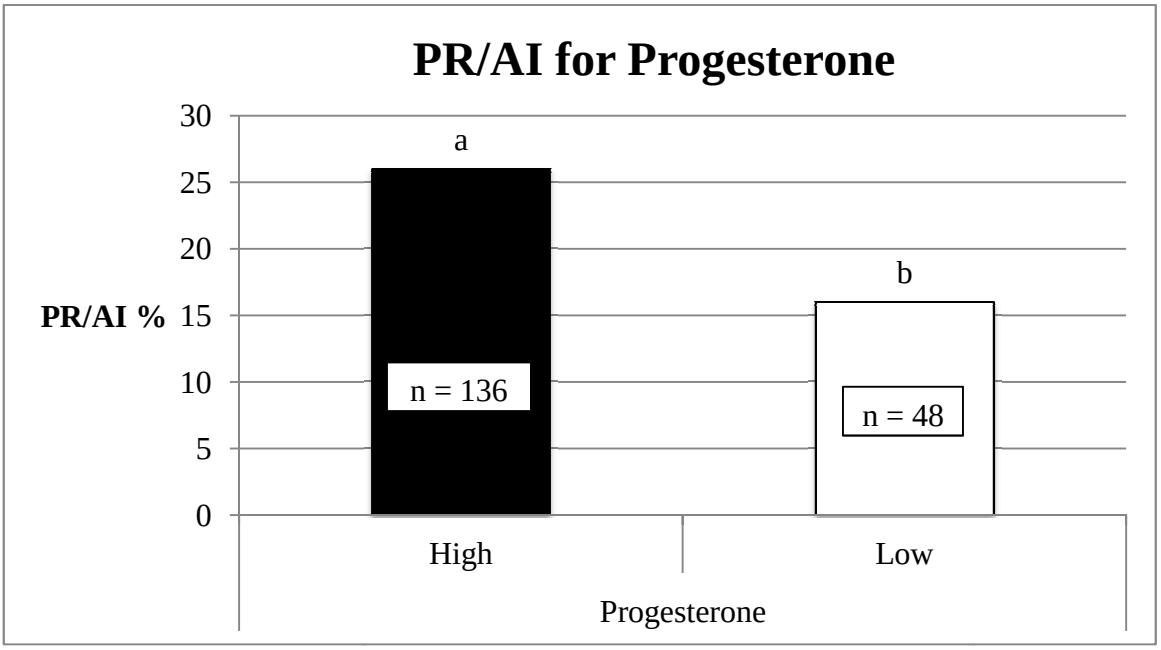

Figure 2.7 Pregnancy rates per artificial insemination (PR/AI) for a subgroup of lactating Holstein cows (n = 184) with high progesterone ($P_4 \geq 1$ ng/mL) or low progesterone ($P_4 < 1$ ng/mL) at initiation of treatment in both treatment groups[∞].

[∞] Day 0 cows were administered gonadotropin-releasing hormone (GnRH) (control) or no GnRH (treatment) and controlled internal drug release (CIDR) inserts were administered to all cows. On day 5 CIDR's were removed and all cows received a prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection. Cows were bred based on heat detection (early; day 6 or 7) and not given the final GnRH or bred timed AI (late; day 8) and given the final GnRH at the time of insemination.

[ab] Bars with different letters tended to be different (P = 0.09).

DISCUSSION

In this study, we examined the effects of the initial GnRH in a 5-day CIDR-Cosynch resynchronization protocol to the second insemination in lactating dairy cows. The majority of dairy producers (87%) in the US use a synchronization protocol for first insemination, usually between 50 to 70 days following parturition (Pursley et al., 1997; Caraviello et al., 2006). The use of TAI protocols have proven to be beneficial in increasing PR/AI, as estrous detection rates are frequently < 50% (Stevenson et al., 1999). Reports have shown that approximately 60% of lactating dairy cows will fail to conceive to first AI, and therefore will be resynchronized on average 32 days following first insemination (Pursley et al., 1997; Jobst et al., 2000; Fricke et al., 2003; Cerri et al., 2004; Chebel et al., 2006; Galvão et al., 2007). Approximately 77% of dairy producers in the US use a systematic breeding protocol for resynchronization, and it has been reported that 69% use Ovsynch for resynchronization (Caraviello et al., 2006).

Pregnancy rate to second and third inseminations is lower than first inseminations. For example it has been reported that second and higher insemination CR are 5 to 10% lower than first inseminations (Fricke et al., 2003; Chebel et al., 2006; Sterry et al., 2007; Bilby et al., 2012; Lucy, 2012), and < 30% of cows resynchronized with Ovsynch upon open diagnosis actually conceived (Bilby et al., 2012). It is not completely clear why PR/AI to Resynch Ovsynch is less than Presynch-Ovsynch but this may be related to biological variation in the stage of the new estrous cycle in cows that have not conceived to the first AI when subjected to resynchronization at approximately 32-40 days after the first AI. This variation results in a variety of ovarian structures at the initiation of a resynchronization protocol, which may reduce the efficacy of resynchronization protocol, especially Resynch-

Ovsynch. It has been well-documented that when an Ovsynch protocol is initiated at random stages of the estrous cycle, 10 to 30% of cows will fail to synchronize to ovulation and have a decreased PR/AI (Vasconcelos et al., 1999). This asynchrony of ovarian structures results in inconsistency in of follicular response to GnRH with ovulation of a dominant follicle. Therefore, the development of a resynchronization protocol, which can synchronize lactating dairy cows to the optimal stage of the estrous cycle at time of AI is critical to optimize PR/AI. Controlled internal drug release inserts have been incorporated into synchronization programs to increase P_4 concentrations prior to administration of $PGF_{2\alpha}$. Many studies have indicated that higher concentrations of P_4 prior to administration of $PGF_{2\alpha}$ result in greater PR/AI (Pursley et al., 1997; Xu et al., 1997; Stevenson et al., 2012b). Progesterone supplementation prevents ovulation by inhibiting gonadotropins (LH/FSH) and may stimulate follicle turnover in cows that did not respond to the initial injection of GnRH in an Ovsynch or Cosynch protocol, also preventing premature luteolysis of an existing CL (Taylor et al., 1991; Savio et al., 1993; Vasconcelos et al., 1999; Xu and Burton, 2000 Lima et al., 2009).

Shortening the length of CIDR treatment from 7 to 5 days decreases the number of days required for a synchronization program and has been shown to have no detrimental effects on overall PR/AI. A shorter CIDR duration would also help reducing the risk of prolonged follicular dominance, thereby avoiding ovulation of an aged oocyte, which reduces embryo quality and PR/AI (Cerri et al., 2004; Bridges et al., 2008; Cerri et al., 2009b; Lopes et al., 2009; Ahmadzadeh et al., 2010; Santos et al., 2010).

PREGNANCY RATES PER ARTIFICAL INSEMINATION

In the current study, there were no differences in milk production, DIMFB, DIMSB, parity, and P_4 concentrations between GnRH and no GnRH treatment groups (Table 2.1), indicating a similar population of animals in each treatment group.

We observed no difference in PR to second AI between lactating Holstein cows given the initial GnRH (control; n = 226; Figure 2.4) or not given the initial GnRH (treatment; n = 203; Figure 2.5) in a 5-day CIDR-Cosynch resynchronization protocol (21.23% GnRH vs. 27.09% no GnRH; Figure 2.6). Given that the same PR/AI can be obtained without the initial GnRH, producers may be able to save $2.50 to $3.50 per cow on resynchronization costs (Stevenson, 2012).

Our findings were similar to other studies, which were conducted in beef and dairy heifers (Howard et al., 2009; Howard et al., 2011; Lima et al., 2011). In these studies, PR/AI were similar between heifers treated with and without the initial GnRH in a 5-day CIDR-Cosynch protocol for the first AI. Similar, in heifers we observed no difference in PR/AI when lactating dairy cows were synchronized for the second insemination using a 5-day CIDR-Cosynch protocol with and without the initial GnRH, further indicating that the initial GnRH may not be necessary. A study by Lima et al., (2013) investigated the effects of the initial GnRH in a 5-day CIDR-Cosynch protocol for first AI as well as one or two injections of $PGF_{2\alpha}$ in dairy heifers and suggested that if initial GnRH was administered, two $PGF_{2\alpha}$ injections may be needed to cause complete luteolysis due to accessory CL's that may have developed. Greater PR/AI were observed when the initial GnRH was administered along with two injections of $PGF_{2\alpha}$ compared to no initial GnRH and either one or two $PGF_{2\alpha}$ injections (Lima et al., 2013). However, it is not known that two

injections of $PGF_{2\alpha}$, specifically in the GnRH group would have resulted in greater PR/AI outcomes in our study.

Potential changes to the existing 5-day Cosynch-CIDR protocol could be as simple as not administering the initial GnRH at the beginning of an Ovsynch or Cosynch resynchronization program. The purpose of the initial GnRH in an Ovsynch resynchronization protocol is to cause ovulation of a dominant follicle. Provided that lactating dairy cows subjected to resynchronization could be at various stages of their estrous cycles at the time of synchronization, a large follicle that is responsive to GnRH may not be present. Therefore, the initial GnRH administration may not be necessary. Moreover, 18 to 27% of dairy cows lack a CL 32-34 days after previous TAI (Fricke et al, 2003; Sterry et al 2007; Bartolome et al., 2009; Bisinotto et al., 2010); hence, they may not be in the optimal stage of the estrous cycle and may not respond to the initial GnRH injection with ovulation and/or luteinization of follicles (Bisinotto et al., 2010). Therefore, it appears that the use of GnRH in a 5-day CIDR-Cosynch resynchronization protocol for the second insemination would provide no additional benefits for increasing PR/AI.

Eliminating the initial GnRH had no negative effect on PR/AI; however, cows in the no GnRH group appear to have greater reproductive efficiency. Cows in the no GnRH group become pregnant earlier as DO was lower compared to the GnRH-treated group. Moreover, no the GnRH group had fewer TBRD (3.68 ± 0.09 GnRH vs. 3.39 ± 0.09 no GnRH; Table 2.2). In this study we observed an average reduction of 13 days on DO (171.71 ± 3.43 days for GnRH vs. 158.30 ± 3.51 days for no GnRH; Table 2.2). There is not a clear explanation as to why the no GnRH group had less DO and TBRD.

PREGNANCY RATES & SERUM PROGESTERONE

The P_4 results indicate that the majority of cows (75.1%) subjected to resynchronization on average of 37 days following first AI had high P_4 (≥ 1 ng/mL; n = 136/184). There was also a tendency (P = 0.09) for cows with P_4 > 1 ng/mL at the initiation of resynchronization to have greater PR/AI than low P_4 (26% high vs. 16% low; Figure 2.7).

Our results are similar to other findings in that P_4 was high (≥ 1 ng/mL) in the majority of cows (> 70%) at initiation of resynchronization 18 to 32 days following first AI (Chebel et al., 2003; Stevenson et al., 2007; Dewey et al., 2010; Green et al., 2011). A study by Chebel et al. (2003) indicated that the initiation of an Ovsynch resynchronization protocol 21 days following first insemination resulted in 72% of dairy cows with > 2.35 ng/mL P_4. Research has indicated that the presence of a CL and higher levels of P_4 concentrations at the initiation of resynchronization protocol resulted in greater PR/AI (Fricke et al., 2003). Given that studies (those referred to above) have indicated that the majority of cows will have high levels of P_4 at the initiation of resynchronization, the initial GnRH injection for resynchronization may not be necessary. In fact, studies have shown that the initial GnRH administration may not cause ovulation of a dominant follicle if P_4 is high at the initiation of resynchronization (Stevenson et al., 2007; Bisinotto et al., 2010).

PREGNANCY RATES BASED ON ESTRUS DETECTION AND PARITY

In this study there was no significant difference between lactating dairy cows bred based on estrous detection (day 6 or 7; n = 50; 28%) or TAI (day 8; n = 379; 23%). Many studies have shown an improvement in PR/AI when cows are bred based on estrous detection either following CIDR removal or 20 to 22 days following first TAI (El-Zarkouny and Stevenson, 2004b; Bisnotto et al., 2010; Santos et al., 2010; Stevenson et al., 2012a).

Although there was a numerical difference of 5% between early and late bred groups in our study, the low number of observations in early (n = 50) limits the power to detect any difference in this study. Similarly, Stevenson et al. (2012a) observed a low proportion of cows that exhibited estrus prior to TAI (15.7%; n = 3,005). Given the low number of cows that were detected in estrus and bred early in our study (n = 50/429) may possibly indicate that shorter CIDR duration treatment may cause a tighter estrous synchronization. This could potentially prove beneficial, as it would limit the number of cows bred before TAI. Our study also observed a significant difference ($P < 0.05$) between parity groups. Pregnancy per AI was significantly higher ($P < 0.01$) in primiparous (28%; n = 119) compared to multiparous (23%; n = 379). These findings are similar to others in that primiparous dairy cows tend to have higher fertility and PR/AI when compared to multiparous (Sterry et al., 2007; Stevenson et al., 2007; Bisinotto et al., 2010; Stevenson et al., 2012a). In fact a study by Stevenson et al (2007) showed a similar difference of 7.6% between primiparous (26.9%) and multiparous (19.3%) animals. The results indicate that primiparous dairy cows have higher PR/AI and fertility compared to multiparous dairy cows.

Although there was no significant difference between no GnRH (n = 203) and GnRH (n = 226) treatment groups (27% vs. 21%) on overall PR/AI, by increasing the sample numbers in each group and increasing the power of a detection, a 6% may become significant in favor of not administering GnRH at the initiation of a 5-day CIDR-Cosynch resynchronization to second AI. By analyzing more P_4 samples at the initiation of this resynchronization protocol may indicate that cows with higher P_4 will result in a greater proportion of cows conceiving than those with low P_4 at the initiation of resynchronization

37 days following first AI. Also given that the herd used for this project has a very high PR (25%), by examining this resynchronization protocol on a less efficient herd more desirable effects may be observed.

CONCLUSION

The initial GnRH in a 5-day CIDR-Cosynch resynchronization program for second AI may not be necessary. This could ultimately save producers $2.50 to $3.50 per cow and decrease the number of DO and number of inseminations required to achieve pregnancy. However, further research is needed in order to determine how applicable this may be to implement into dairy management programs.

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APPENDIX 1

Chapter 1: SAS Tables

Appendix Table 1.1 Analysis of variance for the effect of treatment groups (aspirin vs. control) on plasma progesterone (P_4) concentrations in lactating Holstein cows on day 0 of experimental protocol.

Source	df	Mean Square	F-Value	Pr > F
Treatment	1	0.00002	0.0	0.9798
Cow (Treatment)	2	0.04958	1.13	0.3441
Error	18	0.04377		

Cow within treatment considered random effect.

Appendix Table 1.2 Analysis of variance for the effect of treatment groups (aspirin vs. control) on day to luteolysis in lactating Holstein cows. Day of luteolysis was defined as two consecutive days of < 1 ng/mL progesterone during days 15 to 22 of experimental protocol.

Source	df	Mean Square	F-Value	Pr > F
+ Treatment	1	7.5625	3.17	0.0966
Error	14	33.375		

Cow within treatment considered random effect.
+ Means tended to differ $P \leq 0.1$.

Appendix Table 1.3 Analysis of variance (repeated measure), mixed model using autoregressive moving average (ARMA; 1,1) for the effect of treatment groups (aspirin vs. control), day and treatment by day interactions on plasma progesterone (P_4) concentrations in lactating Holstein cows from day 15 to 22 (n = 8) of experimental protocol.

Source	df	F-Value	Pr > F
Treatment	1	0.20	0.6602
* Day	7	6.67	<0.0001
Treatment × Day	7	0.67	0.7002

Cow within treatment considered random effect.
* Means differ $P \leq 0.05$.

Appendix Table 1.4 Analysis of variance (repeated measure), mixed model using autoregressive moving average (ARMA; 1,1) for the effect of treatment groups (aspirin vs. control), time and treatment by time interactions on plasma prostaglandin metabolite (PGFM) concentrations in lactating Holstein cows from day 14 (before aspirin administration) and six hourly plasma blood samples on day 15 (n = 7) using body weight (BW) as a covariate.

Source	df	F-Value	Pr > F
BW	1	1.54	0.2159
* Treatment	1	5.54	0.0289
* Time	7	6.55	<0.0001
* Treatment × Time	7	3.09	0.0046

Cow within treatment considered random effect.
* Means differ $P \leq 0.05$.

Appendix Table 1.5 Proc Univariate results for prostaglandin metabolite (PGFM) prior to and following log transformation.

Proc Univariate	Skewedness[1]	Shapiro-Wilk[2]
Before Transformation	1.8572	0.7717
After Transformation	0.0596	0.9880

[1] The lower the value the better, < 0.1 indicates stable variance.
[2] The closer to 1 indicates normal distribution of data.

APPENDIX 2

Chapter 1: Animal Care & Use Committee Approval

University of Idaho
Institutional Animal Care and Use Committee

Date: Tuesday, August 19, 2014
To: Amin Ahmadzadeh
From: University of Idaho
Institutional Animal Care and Use Committee
Re: Protocol 2012-133
Effect of Aspirin on Prostaglandin Metabolites and Progesterone Concentrations in Lactating Dairy Cows

Your requested renewal of the animal care and use protocol shown above was reviewed and approved by the Institutional Animal Care and Use Committee on Tuesday, August 19, 2014.

This protocol was originally submitted for review on: Thursday, August 30, 2012
The original approval date for this protocol is: Monday, September 24, 2012
This approval will remain in effect until: Wednesday, August 19, 2015
The protocol may be continued by annual updates until: Thursday, September 24, 2015

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Barrie Robison, IACUC Chair

APPENDIX 3

Chapter 2: Extra Tables

Appendix Table 3.1 Mean ± standard error (SE) for 150 day corrected milk (150CM), days in milk to first breeding (DIMFB), days in milk to second breeding (DIMSB), days open (DO), parity, and number of times bred (TBRD) on occurrence of disease (including mastitis, milk fever, retained placenta, metritis, pneumonia, displaced abomasum, injury, lameness or off feed) within the first 60 days postpartum in primiparous and multiparous lactating Holstein cows.

Response Variable	Disease	No Disease
150CM (kg)	41.69 ± 0.82	40.63 ± 0.60
DIMFB (days)	66.93 ± 1.29	65.37 ± 0.95
DIMSB (days)	112.54 ± 1.34	110.52 ± 0.99
DO (days)	167.76 ± 3.95	162.25 ± 2.91
***** Parity (lactation #)	3.04 ± 0.11	2.25 ± 0.08
TBRD	3.58 ± 0.11	3.49 ± 0.08

Cow within treatment considered random effect.
* Means differ $P \leq 0.05$.

APPENDIX 4

Chapter 2: SAS Tables

Appendix Table 4.1 Logistic regression analysis of the effects of treatment (GnRH vs. no GnRH), day (Early vs. Late), technicians (n = 3), sires (n = 10) and parity (primiparious vs. multiparous) on overall pregnancy rates per artificial insemination (PR/AI) in lactating Holstein cows (n = 429). All two-way interactions with the treatment effects were also included.

Source	df	Wald Chi-Square	Pr > ChiSq
Treatment	1	0.0001	0.9924
Day	1	0.4535	0.5007
+ Technician	2	5.3963	0.0673
Sire	9	3.2521	0.9535
* Parity	1	8.4600	0.0036
Treatment × Day	1	0.1550	0.6938
Treatment × Technician	2	0.4937	0.7813
Treatment × Sire	9	2.9462	0.9664
Treatment × Parity	1	0.0929	0.7605

Cow within treatment considered random effect.
* Means differ $P \leq 0.05$.
+ Means tended differ $P \leq 0.1$.

Appendix Table 4.2 Logistic regression analysis of a subgroup of cows (n = 184) for the effects high progesterone ($P_4 \geq 1$ ng/mL; n = 136) vs. low progesterone ($P_4 < 1$ ng/mL; n = 48) on overall pregnancy rates per artificial insemination (PR/AI) in lactating Holstein cows on day 0 of experimental protocol.

Source	df	Wald Chi-Square	Pr > F
Treatment	1	0.0000	0.9977
Day	1	0.3049	0.5808
* Technician	2	7.7027	0.0213
Sire	8	1.6760	0.9894
+ Progesterone	1	2.7636	0.0964
Parity	1	2.0243	0.1548
Treatment × Day	1	0.3807	0.5372
Treatment × Technician	2	4.2502	0.1194
Treatment × Sire	8	3.4486	0.9031
Treatment × Progesterone	1	1.7497	0.1859
Treatment × Parity	1	1.5929	0.2069

Cow within treatment considered random effect.
* Means differ $P \leq 0.05$.
+ Means tended to differ $P \leq 0.1$.

Appendix Table 4.3 Analysis of variance for the effect of treatment groups (GnRH vs. no GnRH) on serum progesterone (P_4) concentrations in a subpopulation of lactating Holstein cows (n = 184) at the initiation of the experimental protocol.

Source	df	Mean Square	F Value	Pr > F
Treatment	1	13.728	1.20	0.2754

Cow within treatment considered random effect.

APPENDIX 5

Chapter 2: Animal Care & Use Committee Approval

REQUEST FOR RESEARCH/INSTRUCTION
Department of Animal and Veterinary Sciences
5-D Dairy Pasco, WA

Date January 28, 2014

Experiment/Course Title
Fertility of cows following synchronization with a modified 5-day CIDR-$PGF_{2\alpha}$-GnRH timed AI protocol with and without GnRH at CIDR insertion

Project Leader/Instructor Amin Ahmadzadeh

Other Personnel Involved Jennifer Spencer, Dr. Kevin Carnahan, Kathlyn Steinkamp, Courtney Claypool

Date: Beginning November 2013 Ending Spring , 2014

Number and type of treatments (list each):

A. NEEDS

1. Number and type of animal to be used (heifers, calves, lactating cows)
   400 primiparious and multiparious cows for second AI

2. Feed (attach ration if applicable)
   a. Describe ration to be used. Include any specialty feeds to use and expected cost.
      N/A The ration s ispreparedby 5-D farms
   b. What standard ration will be replaced, if any?
      N/A
   c. What individual or special feeding/weigh backs are required.
      N/A

4. Any unusual physical requirements associated with feed handling, preparation, storage, waste handling, animal housing, etc.
   The free stall lock up will be used for ultrasonography, blood sampling and estrous synchronization.

   On day 0, 5, 8, and 40 cows will be locked in headlocks and subjected to resynchronization protocol and blood sampling.

Farm equipment needed.
N/A

Other special requirements.
~~***Treatment: The treatment group (n=200) will be not given a GnRH injection and the control group (n=200)***~~ *will be given a GnRH injection at the initiation of the resynchronization protocol. Initiation of resynchronization will be considered day 0 and all cows will receive their treatment and we will insert a CIDR (d 0). Five days following the CIDR insertion, we will remove the CIDR and give a single injection of $PGF_{2\alpha}$, subsequently 60-72 hrs after we will give all cows GnRH and they will be subjected to timed AI. Coccygeal blood samples will be collected on days 0 and 8 for measurement of P4 to determine the status of the estrus cycle. Coccygeal blood samples will also be taken on day 40 to determine pregnancy by BioTracking Inc.*

RESPONSIBILITIES

1. Routine (5-D Farms personnel responsible for all activities unless otherwise noted)
   a. Feeding (*Will be done by 5-D Dairy crew*)
   b. Cleaning (*5-D Dairy crew*)
   c. Animal health/care *(will be monitored by herd manager and herd health veterinarian and recorded and treated following 5-D protocol)*
   d. Breeding *(AI will be preformed by 5-D certified AI technition)*
   e. Records
      All records will be obtain fron the dairy record software located on the farm (Daircomp 305)
   f. Management *(All cows will be managed by the owner, Steve DeRuetr, and Dr. Jaime Andrade, the herdmangerof 5-D Farms*

2. Experimental/Instructional (Investigator/Instructor is responsible; please indicate who will do the work.)
   a. Individual or special feeding/weigh backs *Cows will be fed by dairy*
   b. Sample collections/milk weights, etc. *Blood sampels collection will be done by the Investagatior and other UI personnel involved*
   c. Health care Will be conducted by herd health veterinarian (Dr. Kieser) and *dairy superintendent. In case of any observed health issue, the dairy superintendent will be informed. All animal treatments will be done by the 5-D Farms staff.*
   d. Breeding *Will be preformed by 5-D AI technition and pregnancy check will be done by Dr. Kiesler*
   e. Records *Managed by 5-D herdsman*
   f. Management *General chore and regular routin management is done by the dairy crew.*
   g. *All the drugs are provided free ofcharge hy the PI and under supervision of the 5-D Farms herdhhealth veterinarian*

METHOD OF EUTHANASIA

In the case emergency and at the discretion of the herd's veterinarian, cows may be euthanized using the the 5-D Farms protocol under supervisionof hed healt veteerianrian and 5-D Farms Manager.

C. SURGICALLY MODIFIED ANIMALS

1. Does your project involve surgically modified animals? Yes_____ No___X___ (If Yes, provide details)

D. FINANCIAL IMPACT

1. Please estimate per diem charges for this project.
   In agreement with the farm owner (Mr. DeRuyter), there will be no per diem charges.

2. Please estimate higher feed cost, III help, health care, and other experimental/instructional costs associated with the project to be charged to research or instructional budgets.
   No anticipated higher feed, health car, etc. All the hormes costs are covered by the PI

3. Please estimate costs associated with loss of milk or receipts associated with termination of animals due to instructional or research protocol (number of animals × loss/animal), if applicable.
   No loss of milk is anticipated with the experiment. If any cow unexpectedly is terminated due to this experiment, the cost wil be covered by the investigator.

4. Please estimate cost of retaining cull or mature animals beyond normal time, or purchase of special animals, if applicable.
   N/A
5. Please estimate financial loss/gain from purchase/resale of animals, if applicable.
   N/A
6. Estimated total cost/charges for this project.
   There is not charge associated with this research.

Jaime Andrade

PRINCIPAL INVESTIGATOR ASSURANCE

The information contained on this form provides an accurate description of the animal care and use protocol which will be followed. I agree to abide by governmental regulations and university policies concerning the use of animals. I will allow veterinary care to be provided to animals showing evidence of pain or illness. If the information provided for this project concerning animal use should be revised, or procedures changed, I will so notify the committee of those changes. All proposed changes will not be implemented until full IACUC approval has been granted. I understand that failure to report significant changes may place the university and myself in violation of federal regulations.

As required by federal regulations, *the activities described do not unnecessarily duplicate previous experiments.*

11/4/2013

Signature of Principal Investigator — Date

Send original form plus seven copies to the University Research Office, 111 Morrill Hall (885-6651)

Jaime Andrade