EFFECT OF HUMAN CHORIONIC GONADOTROPIN (hCG) POST-OVULATION ON TIME TO CONCEPTUS ATTACHMENT IN LACTATING DAIRY COWS

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ABSTRACT

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Increasing progesterone (P4) during conceptus development is crucial for establishment of pregnancy in dairy cattle. We developed a robust technique to estimate d of conceptus attachment (CA) based on daily within-cow changes in concentrations of pregnancy-specific protein B (PSPB; BioPRYN). Highest sensitivity and specificity were obtained with a 12.5% increase in addition to 2 consecutive d of continuous increases when predicting CA. We hypothesized that increasing serum P4 post-ovulation would favor embryonic development and reduce time to CA in lactating dairy cows. The first objective was to determine the effects of treatment with hCG on d2, d5, and d2 and 5 post-ovulation on luteal function and serum P4. Treatment with hCG on d 2 & 5 or on d 5 increased P4 by inducing accessory CL (aCL) and increasing the volume of existing CL. On d 2, hCG increased P4 on d 5 post-ovulation. The second objective was to investigate the effects of treatment with hCG on time to CA. Treatment on d 5 reduced the percentage of cows with CA and increased time to CA. Primiparous cows benefited from ipsilateral aCL against pregnancy loss before d 100 post-AI/ovulation. Delayed time to CA was associated with pregnancy losses before d 35. The highest quartile of P4 on d 5, but not on d 19 & 20, was associated with reduced time to CA. Our hypothesis was not confirmed. Yet, early serum P4, but not late, was associated with time to conceptus attachment in multiparous dairy cows. Our findings indicated that treatment with hCG post-ovulation was not financially advantageous as treatment on d 5 or aCL induced by treatment reduced annual herd profits compared to standard reproductive programs such as Double-Ovsynch and Ovsynch.

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KEY TO ABBREVIATIONS

aCL	accessory corpora lutea
AI	artificial insemination
BCS	body condition score
BNCs	giant binucleate cells
C	Celsius
CA	conceptus attachment
CL	corpora lutea
cm	centimeter
CV	coefficient of variation
d	days
D	diameter
DIM	days in milk
DO	Double-Ovsynch
EB-FTAI	Estradiol-based fixed-time AI
ELISA	enzyme-linked immunosorbent assay
ES	estrus synchronization
ET	embryo transfer
FSH	follicle-stimulating hormone
g	*g* force
GCP-2	granulocyte chemotactic protein-2
GH	growth hormone

GnRH	gonadotropin-releasing hormone
h	hours
hCG	human chorionic gonadotropin
HRP	horseradish peroxidase
IFNT	interferon-τ
IL	interleukin
IRF-1, -2	interferon regulatory factor
ISGs	interferon-stimulated gene
IU	international unit
kDa	kilo Dalton
kg	kilogram
lb.	pound
LH	luteinizing hormone
M	multiparous
MHz	megahertz
min	minutes
mL	milliliter
mm	millimeter
mm^3	millimeter cubed
mRNAs	messenger ribonucleic acid
Mx-1,-2	*Myxovirus*-resistant gene-1 and -2
n	sample size
NE	natural estrus

ng	nanogram
nm	nanometer
OD	optical density
OF	ovulatory follicle
Ov	Ovsynch
P	primiparous
P/AI	pregnancies per artificial insemination
P4	progesterone
PAGs	pregnancy-associated glycoproteins
PBL	peripheral blood leukocytes
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
pH	potential of Hydrogen
PL	prolactin
Pre-Ov	Presynch-Ovsynch
PRID	progesterone-releasing intravaginal device
PRP	prolactin-related proteins
PSMG	pregnant mare serum gonadotropin
PSP-60 kDa	pregnancy specific protein-60 kilo Dalton
PSPB	pregnancy specific protein-B
R	radius
RIA	radioimmunoassay
ROC	receiving operating characteristic

RT-PCR	reverse transcription-polymerase chain reaction
SEM	standard error of the mean
Th1	T helper type 1 cells
Th2	T helper type 2 cells
TMR	total mixed ration
TSH	thyroid stimulating hormone
USD	United States dollar
UTJ	uterine-tubular juction
V	volume
β2-MG	β2-microglobulin
µg	microgram

CHAPTER 1

REVIEW: CONCEPTUS DEVELOPMENT AND ESTABLISHMENT OF PREGNANCY IN DAIRY CATTLE

INTRODUCTION

Early embryonic development and attachment in eutherian mammals involve many delicate and yet complex physiological events (Maalouf et al., 2014). Timely progression of these events is fundamental for establishment and maintenance of pregnancy (Chang, 1952). Yet, considerable incidence of pregnancy losses occur during the first trimester of pregnancy in dairy cattle (Wiltbank et al., 2016).

It is estimated that 20 to 50 % of cases of embryonic death occur during the first week of development (Sartori et al., 2002; Cerri et al., 2009). Also, studies suggest that approximately 30 % of total pregnancy losses occur between d 8 and 27 (Han et al., 2006; Monteiro et al., 2014). Most of these have been attributed to failures in the process of maternal recognition of pregnancy or during embryo-maternal interactions leading to failure in conceptus attachment (Wiltbank et al., 2016). Therefore, this literature review aims to present what is currently known about early embryonic development leading up to conceptus attachment in dairy cows, the physiology associated with these events, and strategies to prevent early embryonic death.

DEVELOPMENT OF THE BOVINE CONCEPTUS

Fertilization and the pre-hatching embryo

Time seems to be a crucial component of physiological events leading to pregnancy even before fertilization occurs. In cattle, the oocyte is typically fertilized in the oviduct shortly after ovulation and sperm capacitation (Miller et al., 1931). Early studies on oocyte viability and fertilization confirmed that oocytes have a short lifespan, 6 to 10 hours, relative to the time of ovulation from the ovary (Trimberger, 1948; Szollosi, 1973). Oocyte nuclear instability and limited cytoplasmic organelles have been associated with such short lifespan (Szollosi, 1973;

Hunter, 1985). With the discovery of protocols for synchronization of ovulation by Pursley et al. in 1995, scientists were able to confirm that delayed time to insemination relative to ovulation allows for aging of the oocyte leading to failure in fertilization and limited embryonic development in dairy cows (Pursley et al., 1998).

Following successful fertilization, the early developing conceptus is transported towards the uterus while undergoing its first series of mitotic divisions. It travels through the oviduct for approximately 4 d entering the uterus at the 16-cell stage of embryonic development (Miller et al., 1931). As development progresses, the conceptus grows into a compact cellular mass, named morula, which is soon followed by the blastocyst stage. At this stage, a fluid filled cavity (blastocoele) can be observed as cells separate from the trophectoderm, outer single-cell layer that surround a tightly attached inner cell mass (Winters et al., 1942; Hamilton and Laing, 1946; Melton et al., 1951; Lonergan et al., 2016).

At the pre-hatching stage, the bovine blastocyst initiates conceptus-maternal interactions via local and systemic immune pathways (Maeda et al., 2013). This level of communication has gained significant attention among scientists in the past decades as embryonic mortality at this point of development has been associated with high milk production in dairy cattle. Recent observations indicate that conceptus-maternal interactions at the pre-hatching stage primarily involve the activation of anti-inflammatory pathways via T helper type 2 cells (Th2) as foundation of immune tolerance to the semi-allogenic conceptus in the oviduct (Maeda et al., 2013). Th2 cells are commonly known for producing interleukins (IL) 4, 5, and 10 and down-regulating T helper type 1 cells (Th1) inflammatory pathways. The Th1 inflammatory immune response is usually triggered as a first response to sperm components entering the uterus after breeding. Th1 response endangers pre-hatching embryo development, making Th2 anti-

inflammatory response critical for the development (Adkins et al., 2004; Talukder et al., 2020). Such anti-inflammatory response is normally induced via initial secretion of interferon-tau (IFNT) by the pre-hatching embryo (Rashid et al., 2018).

Evidence of the early release of IFNT by the pre-hatching embryo have been demonstrated. Shirasuna et al. (2011) revealed that pre-hatching embryos are capable of synthesizing and releasing IFNT. These authors revealed greater expression of interferon-associated genes (ISGs) on samples from pregnant cows on d 5 compared to d 8 samples of both confirmed pregnant and non-pregnant cows. Similar findings have been demonstrated by Yaginuma and co-authors (2019), who have revealed increased expression of a similar set of ISGs in maternal immune cells of Holstein cows and heifers as a result of sham-intrauterine infusion using cultured conditional media (TCM-199) from 8-day *in-vitro* maturation of bovine embryos.

Pre-hatching embryos have also been recognized for actively inducing modifications to the endometrial transcriptome and the composition of the uterine luminal fluid (ULF) or histotroph. When measuring the of the abundance of specific transcripts in different regions of the 7-day pregnant uterine horn, Sponchiado et al. (2017) reported greater expression of ISGs at the uterine-tubular junction (UTJ) of pregnant cows compared to sham-inseminated cows. In a similar study, Sponchiado et al. (2019) confirmed that the presence of a pre-hatching embryo in the uterus also induces changes in the composition of the histotroph. These authors measured the abundance of 167 different metabolites in the histotroph of pregnant and sham-inseminated cows and concluded that presence of 7-day embryos were associated with decreased concentrations of histotroph compounds compared to non-pregnant cows. These compounds include mainly those in the classes of phospholipids, acylcarnitines, amino acids, and biogenic amines. These findings

confirm that bovine embryos actively engage in the conceptus-maternal signaling at the pre-hatching stage inducing changes in the uterine microenvironment to support further embryonic development.

Hatching

The blastocyst hatches from the zona pellucida between d 8 and 11 post-fertilization as it develops in the uterus. This process was characterized by an active rupture of the zona pellucida trigged by expansion of the blastocyst (Betteridge and Fléchon, 1988). It has been reported by Chang (1952) that this process involves shrinking of the embryonic cell mass followed by loss of zona pellucida within 10 to 11 d of embryonic development. Similarly, Massip et al. (1982) showed evidence that hatching was initiated by uninterrupted blastocyst expansion and that such expansion depends upon its ability to retain and accumulate fluids inside the blastocele. Menino and Williams (1987) later demonstrated that hatching also involves the activation of plasminogen to the enzyme plasmin to drive the rupture and escape from the zona pellucida in parallel with blastocyst expansion.

The hatched blastocyst re-expands and changes from a spherical to ovoid shape between d 10 and 14 of development. This change in shape precedes subsequent elongation (Chang, 1952; Betteridge et al., 1980). At this time, the establishment of a reliable maternal-conceptus relationship becomes more essential as the hatched blastocyst depends entirely on uterine histotrophic nourishment for survival and to advance in development. Such dependence was associated with changes in the embryo's metabolic and energy requirements necessary to sustain development (Betteridge and Fléchon, 1988). Embryotropic nutritional substances can only be provided by the histotroph (Fléchon et al., 1986).

Elongation

The expanded blastocyst starts to elongate from an ovoid to filamentous shape. Such change in shape and size can be observed soon after blastocyst expansion as early as d 9 of development. Numerous studies have reported that the uterine environment is necessary for the conceptus to achieve filamentous elongation (Betteridge and Fléchon, 1988; Gray et al., 2001; Brandão et al., 2004). This limitation was also linked to the conceptus' dependence on embryotropic compounds released by endometrial gland in the form of the histotroph (Gray et al., 2001; Brandão et al., 2004; Forde et al., 2011) and cannot be repeated within an in vitro environment.

Filamentous elongation involves changes in genomic and cellular content of the trophoblast leading to exponential growth (Betteridge et al., 1980; Grealy et al., 1996; Hue et al., 2012). Total length of the blastocyst doubles daily as elongation progresses between d 9 and 16 of conceptus development and an added acceleration in growth was reported between d 13 and 14 (Chang, 1952; Betteridge et al., 1980). This expansion of the trophectoderm was required for later conceptus attachment and placentation as the trophectoderm is then put in close apposition to the endometrium and will differentiate into the functional placenta (Chang, 1952; Betteridge et al., 1980; Berg et al., 2010; Hue et al., 2012).

Several findings have reported a wide variation in length of elongated concepti even when these are retrieved from a single donor. A hypothesized explanation suggests that such variability is related to different growth rates among embryos (Betteridge et al., 1980). Berg et al. (2010) has demonstrated that embryos retrieved from beef heifers grow at a faster rate than those retrieved from parous cows. These authors showed evidence that embryos incubated and retrieved from heifers were twice the size of those obtained from cows. However, a greater

variation in size of conceptus was also observed in heifers compared to cows. One of the hypotheses raised by these authors proposed that such variation between heifers and parous cows was associated to lactational stress. On that account, this relationship may suggest that differences in growth rate of elongating conceptus between heifers and cows may be due to differences in levels of P4 (Sartori et al., 2002, 2004). This link between differences in levels of P4 and conceptus elongation will be explored in depth further in this review.

The elongating blastocyst provides signaling necessary to determine the fate of the CL formed after ovulation. In cyclic cows, the process of luteolysis marks the end of the estrous cycle allowing for maturation and ovulation of a new dominant follicle. Without the presence of a conceptus in the uterus, luteolysis was induced by increasing pulsatile release of prostaglandin F2α ($PGF_{2\alpha}$) from the endometrium after a complex cascade of events unfolds between d 17 and 20 post-ovulation (Goding, 1974; McCracken et al., 1984). Increasing concentrations of IFNT can be observed soon after hatching, reaching maximum levels around d 19 after fertilization (Bartol et al., 1985). Elongating blastocysts synthesize greater amounts of IFNT and other components associated with maternal recognition of pregnancy compared to pre-hatching embryos (Mann and Lamming, 2001). IFNT is synthesized by trophoblastic cells. It has antiluteolytic properties that prolong lifespan of the CL by inhibiting the activation of oxytocin receptors and inhibiting the synthesis and secretion of $PGF_{2\alpha}$ (Garrett et al., 1988b; Robinson et al., 1999). Hence, maintenance of CL and progression of pregnancy depends on signaling provided by the hatched, elongating blastocyst (Garrett et al., 1988a; Mann and Lamming, 2001).

Timely synchrony between conceptus development and the maternal estrous cycle becomes crucial for successful pregnancy due to the relationship between the maternal estrous cycle and conceptus signaling in the post-hatching period (Rowson et al., 1969). This synchrony

was required to ensure that the maternal reproductive system can effectively interpret signaling from the conceptus as well as properly provide nourishment to the developing conceptus through the histotroph (Betteridge et al., 1980). Studies on embryo transfer (ET) in both cattle (Hasler, 2001) and sheep (Moore et al., 1963) have demonstrated that disparities of $\leq$ 48 h between day of the estrous cycle and age of the conceptus are acceptable for progression of pregnancy. However, these studies have shown that greater pregnancy rates were observed when ET was performed with no more than 24 h of asynchrony between the two. This relationship was linked to actions mediated by P4 on the endometrium that favor conceptus development (Lawson and Cahill, 1983; Garrett et al., 1988a). The relationship between conceptus development and P4 will be discussed further in this review.

BIOLOGY OF CONCEPTUS ATTACHMENT

Scientists have been conflicted when selecting the most adequate terminology to describe the process of conceptus attachment in cattle. The terms ‘implantation’ and ‘attachment’ have been commonly used interchangeably. Still, the term ‘attachment’ (e. g. King et al., 1980; Spencer et al., 2017) has been preferred over the term ‘implantation’ (e. g. Chang, 1952) when referring specifically to bovine embryos due to the nature of such process. Peippo et al. (2011) argued that bovine embryos never fully penetrate the endometrial epithelium when establishing physical contact with the uterine wall. Differently from humans, this process was described as limited superficial placentation in which only fetal membranes (the trophectoderm) appear to adhere to the endometrium as placentation takes place in cattle (Winters et al., 1942). For this reason, this literature review will use the term ‘conceptus attachment’ to denote to such process.

Early morphological studies have described the process of conceptus attachment as a gradual and continuous process that involves close apposition and non-invasive adhesion of the embryonic trophectoderm to the endometrial epithelium (Chang, 1952; King et al., 1980; Wooding and Wathes, 1980). These studies demonstrated that conceptus attachment begins around d 20 to 22 after fertilization with close proximity between the trophectoderm and the endometrial epithelium (apposition) in some areas of the maternal-conceptus interface, also known as caruncles. At this time, the apical surface of endometrial epithelial cells on caruncles appear to develop protrusions and the endometrial epithelium becomes enlarged, which has also been described as a local epithelial edema. On the conceptus, trophectoderm cells proliferate, and cellular protrusions temporarily disappear differentiating their surface into a smooth arrangement in preparation for conceptus attachment (Melton et al., 1951; Guillomot et al., 1981; Guillomot and Guay, 1982; Yamakoshi et al., 2012).

Soon after close apposition of the trophectoderm and endometrial epithelium at the maternal-conceptus interface, maternal microvilli project themselves into the apical margins of the trophectoderm cells. This is later accompanied by mutual interdigitation of microvilli from both the trophectoderm and endometrial epithelial cells towards one another (King et al., 1980). Interdigitation of epithelia, known as the adhesion stage, assists and precedes conceptus attachment. It provides support to the process of conceptus attachment by anchoring the conceptus to the endometrial epithelium and by providing a route of access to glandular secretions from the endometrial epithelium (Guillomot et al., 1981). The adhesion between trophectoderm and endometrial epithelium triggers differentiation of trophectoderm cells into what is known as giant binucleate cells (BNCs; Guillomot and Guay, 1982). These BNCs rise from mitotic polyploidy of trophectoderm cells that undergo nuclear division followed by failure

in cytokinesis (Wooding, 1982). BNCs migrate and fuse with endometrial epithelial cells forming a transient multinucleated syncytium between d 20 and 21 post-breeding. During this migration and fusion, conceptus attachment is established (Guillomot et al., 1981). This level of adhesion between trophectoderm and endometrial epithelium, alongside with the temporary formation of a syncytium, is a hallmark of the type of epitheliochorial placentation that occurs in bovine (King et al., 1980; Guillomot et al., 1981).

The formation of a syncytial multinucleated cellular structure between trophectoderm and endometrial epithelium (symplasm) facilitates maternal-conceptus communication throughout pregnancy. The symplasm exerts secretory functions and plays crucial role in the exchange of compounds between both tissues (Guillomot et al., 1981). Wooding (1982) discussed that the main function of the symplasm is to provide the conceptus with a general method of delivery of large molecules into the maternal circulation. It has been demonstrated that mature BNCs have large cellular volume mainly composed of cytoplasmic granules (Guillomot et al., 1981; Wooding, 1992). During migration and fusion with the endometrial epithelium, BNCs differentiate into trinucleate cells and release a significant amount of their granules and cytoplasm into the maternal tissue and circulation. These secretory granules contain many glycoproteins such as pregnancy associated glycoproteins (PAGs), placental lactogen (PL), and the prolactin-related proteins (PRP) that are essential for progression of pregnancy inside and outside of the reproductive system (Duello et al., 1986; Sasser, 1986; Green et al., 2000; Patel et al., 2004).

PREGNANCY ASSOCIATED GLYCOPROTEINS (PAGs) AND PREGNANCY-SPECIFIC PROTEIN B (PSPB) AS MARKERS OF CONCEPTUS ATTACHMENT

As discussed, PAGs are one of the many glycoproteins contained within secretory granules of BNCs. Based on phylogenetic and challenge studies, PAGs are described as members of the aspartic proteinase family for avidly binding to aspartic proteinase inhibitors such as pepstatin A at neutral pH (Xie et al., 1991; Hughes et al., 2003; Wooding et al., 2005). Phylogenic analysis of amino acid sequence of PAGs are evidence that these glycoproteins form two distinct groups: one with ancient evolutionary origins and another modern group that has been recently discovered. Greater level of similarity and activity of proteinases at low pH was observed among ancient PAGs while alterations in their catalytic centers have been suggested to prevent modern PAGs from the ability to bind to substrate and act as proteinases (Telugu et al., 2010).

Expression of PAGs exhibit a wide range of patterns throughout pregnancy in ruminants (Xie et al., 1991). An interesting investigation on temporal distribution of expression of different PAGs was published by Green et al. (2000). These authors used Northern blotting and relative expression of mRNA for specific PAGs to estimate the temporal variation in expression of PAGs in bovine placental tissue throughout pregnancy. Although Northern blotting indicated that both PAG-1 and -9 were indeed expressed in placental samples of early pregnancy (d 25), the more precise measurement of relative expression of PAG mRNAs (RT-PCR) suggested that PAG-1 was not at all expressed during that same period of gestation. Relative expression of both PAG-1 and -9 seemed to decline while others were continuous as pregnancy progressed. With regards to their spatial expression, mRNA for PSPB (or PAG-1), as well as for most of its phylogenetically associated and modern counterparts (PAG-14, -15, -16, -17, -18, -19, -20, and -2), was

predominantly discovered within bovine BNCs. mRNA for ancient PAGs were, on the other hand, restricted the outer trophectoderm cells (Green et al., 2000; Wooding et al., 2005).

Increasing levels of pregnancy specific protein-B (PSPB) in the maternal circulation have been observed concomitant with the migration and fusion of BNCs to the endometrial epithelium (Sasser, 1986; Mialon et al., 1993; Wooding et al., 2005). For this reason, PSPB has been widely studied in the past decades for its high potential as a method to diagnose pregnancy in ruminants (Green et al., 2000; Patel et al., 2004; Pohler et al., 2016; Middleton and Pursley, 2019). Since its discovery, this glycoprotein has been referred to as PSPB (Butler et al., 1982; Sasser, 1986), PAG-1 (Xie et al., 1991), and PSP-60 kDa (Mialon et al., 1993). The first study to characterize and identify PSPB in the blood of pregnant cows was published by (Butler et al., 1982). It followed a succession of previous studies that had demonstrated the presence of similar proteins in uterine flushing of pregnant cows (Roberts and Parker, 1976) and ewes (Roberts et al., 1976).

Because of its considerable biological properties such as robust binding and adhesive capacity, immunological camouflage, and high stability within maternal tissues, studies have argued that PSPB may be involved in important functions related to immunomodulatory pathways and luteotropic effects during the time of maternal recognition of pregnancy and conceptus attachment (Green et al., 2000; Wooding et al., 2005). Interestingly, studies from the early and mid 90s published by Del Vecchio's group demonstrated that PSPB increases the concentration of prostaglandin E_2 (PGE_2) and P4 in the culture media from *in-vitro* cultured luteal and endometrial epithelial cells (Del Vecchio et al., 1990, 1995, 1996). Similarly, Weems' group reported comparable outcomes obtained across three studies indicating that PSPB, when added to the culture media, increases synthesis and release of P4 and PGE_2 from *in-vitro* cultured endometrial epithelial cells of pregnant ewes on d 60 and 90 post-breeding and from *in-vitro*

cultured bovine luteal cells (Weems et al., 1998a; b, 2003). Furthermore, in a unique study examining the interaction between PAGs, other chorionic products, and bovine gonadal and extragonadal binding sites, Szafranska et al. (2007) demonstrated that tissues containing LH receptors interact with chorionic proteins. Even though this later study did not characterize or identify specific PAGs, it is an indicator of interactions between uterine receptors and multiple PAGs that may include PSPB.

In parallel, data suggests that PAGs, PGE_2, and P4 is involved in immunosuppressive actions on endometrial epithelium, leukocyte, and myeloid cells of humans, cows, and ewes (Fujisaki et al., 1982; Low, Boon G.; Hansen, 1988; Hoeben et al., 1999). In these reports, high concentrations of PGE_2 and P4 added to the media at the beginning of the culture inhibited proliferation and modified the response of lymphocytes *in vitro* (Fujisaki et al., 1982; Low, Boon G.; Hansen, 1988). Correspondingly, when investigating the effect of bovine PAGs on the proliferation of myeloid hematopoietic cells *in vitro*, Hoeben et al. (1999) observed a 29 % decrease in growth of bovine myeloid and erythroid cell lineages after treatment with 2,400 and 3,000 ng/mL of bovine purified PAGs. In parallel, Austin et al. (1999) tested the effect of different concentrations of PSPB obtained from 120-day cotyledons on the synthesis of uterine proteins by cultured endometrial explants. Their study provided evidence that PSPB induces synthesis of at least one alpha chemokine (granulocyte chemotactic protein-2; GCP-2) which is normally released in response to IFNT and has an anti-inflammatory role during the establishment of pregnancy.

Other studies suggest that PAGs/PSPB may exert limited immunosuppressive actions on endometrial epithelium, leukocyte, and myeloid cells through association with uterine serine proteinase inhibitor (serpins). This has been linked to the confirmation that PAGs/PSPB can

naturally bind to uterine serpins having their proteinase activity neutralized (Mathialagan and Hansen, 1996; Telugu et al., 2010). Wooding et al. (2005) discusses that PAGs could potentially participate in the series of events leading to parturition and release of the cotyledon from the caruncle because of its intriguing proteolytic activity at low pH. Although studies investigating similar effects *in vivo* are lacking, it seems that PSPB and PAGs may have immunosuppressive and luteotropic effects on the endometrial epithelium and the CL that may occur after conceptus attachment has been established in ruminants.

Many studies have investigated the application of PSPB as a marker for embryo viability and pregnancy in ruminants (Semanbo et al., 1992; Green et al., 2000; Patel et al., 2004; Pohler et al., 2016; Middleton and Pursley, 2019). The first method that measured PSPB in serum of pregnant cows was developed by Sasser (1986). These authors developed a radioimmunoassay (RIA) using antisera for PSPB with minimal or no cross-reactivity with bovine pituitary hormones (thyroid stimulating hormone, TSH; growth hormone, GH; and PL), chorionic hormones (hCG; and pregnant mare serum gonadotropin, PSMG), or gonadotropin hormones (LH, and follicle-stimulating hormone, FSH). Their novel method was able to detect PSPB in pregnant cows as early as d 15 of gestation in some cows but d 24 in most of inseminated cows. Detection of PSPB on d 24 was predominantly followed by continuous increase throughout pregnancy with no PSPB detected in nulliparous heifers, indicating that PSPB increased in response to the presence of a conceptus in the uterus. Their study also was evidence that a rapid rise in serum PSPB within the final weeks of gestation was followed by a progressive decrease during the post-partum period. Using the method developed by Sasser (1986), Kiracofe et al. (1993) estimated that the half-life of PSPB ranges from 7.1 to 8.5 d in the circulation of post-partum beef cows, with a mean concentration of 1,209 ng/mL among untreated beef cows on the

first d post-partum. Their findings suggested that PSPB decreases faster in hysterectomized beef cows compared to untreated cows. According to their regression estimate, PSPB was predicted to become undetectable within 68.1 d in hysterectomized cows compared to 87 d in untreated cows.

Later studies have tested the application of RIA for PSPB for pregnancy diagnosis. Zoli et al. (1992) compared pregnancy diagnosis using RIA for PSPB on a single blood sample on d 35 of gestation vs. rectal palpation on d 45. This study used a threshold level of > 0.5 ng/mL in serum PSPB to estimate pregnancy. It reported a 93.03 % accuracy in detecting pregnancy, 97.9 % in detecting nonpregnancy, and an overall accuracy of 94.65 %. These authors justified the 6.97 % of false positives on the occurrence of pregnancy loss between blood sampling on d 35 and the time of rectal palpation and attributed the 2.1 % false negative diagnosis on the wide variation in serum PSPB between animals. Their results demonstrated that serum PSPB could be used for detection of pregnancy by d 35 with high accuracy. That same year, Semanbo et al. (1992) investigated the use of serum PSPB and P4 as tools to monitor embryonic viability during early pregnancy (from d 30 to 41) in cows experimentally infected with *Actinomyces pyogenes* and cows that had abortion induced with $PGF_{2\alpha}$. In their study, all pregnant cows presented high concentration of serum PSPB prior to treatment, ranging from 1.7 to 2.8 ng/mL on the day of treatment. The percentage difference in serum PSPB within cow started to decrease steadily within the first 24 h of inoculation in both groups. Serum P4, however, had considerably different patterns between inoculated and treated groups. Amongst inoculated cows, serum P4 maintained high concentration while a significant decline was observed in those cows treated with $PGF_{2\alpha}$. This study confirmed that the decreasing profile in serum PSPB is highly correlated with pregnancy loss in addition to its increasing profile observed during establishment of pregnancy reported by Zoli et al. (1992).

More recently, Green et al. (2005) developed an enzyme-linked immunosorbent assay (ELISA) to measure serum concentration of early pregnancy PAGs. These authors produced a mixture of monoclonal and polyclonal anti-PAG antibodies that allowed binding and measurement of different epitopes in common with PAG-1 occurring between d 24 and 34. Their rationale is associated to the pattern of expression identified by Green et al. (2000) that indicated that other PAGs (as PAG-4, -5, -8, -9, -10, and -11) were expressed by BNCs on d 25 of pregnancy while PAG-1 was not. The ELISA assay proposed in their study allowed unequivocal detection of pregnancy in all cows by d 28 and in the majority by d 25 of gestation through the identification of PAGs with relative short half-lives. The serum profiles of PAGs reported by these authors were similar to those described in previous studies using RIAs (Sasser, 1986; Semanbo et al., 1992; Zoli et al., 1992; Kiracofe et al., 1993). Such profiles included the detection of PAGs as early as d 22 of pregnancy, the rapid rise in serum concentrations during early pregnancy, continuous increase in concentrations, followed by a dramatic rise prior to parturition reaching a peak within days of calving. Additionally, their study confirmed the existence of a wide variation in serum concentration of PAGs between cows, which seems to agree with several other studies that also used cut points in serum PAGs to determine and diagnose pregnancy (Sasser, 1986; Zoli et al., 1992; Kiracofe et al., 1993).

A later study compared the accuracy of the PAG ELISA developed by Green et al. (2005) to transrectal ultrasonography (Silva et al., 2007). A substantial sample size of 1,673 lactating Holstein cows timed-inseminated after Presynch and Ovsynch programs (1st service) or Ovsynch ($\geq$ 2nd services) was used to compare methods of pregnancy diagnosis in cows on d 27 d after AI. These authors reported 95.4 % sensitivity and 94.2 % specificity for detection of pregnancy using PAG ELISA assay in comparison to ultrasonography. Their findings demonstrated

substantial variation in serum concentration of PAGs on d 27 between cows enrolled in the study. Even though this assay provides a significant level of accuracy, a difference of 4.6 percentage points between methods of pregnancy diagnosis would result in pregnancy losses during the resynchronization protocol. Hence, these authors argue that later samples (i.e., d 30) would provide greater sensitivity due to the continuous increase in concentration of PAGs in maternal circulation with progression of pregnancy.

In 1993, bioTRACKING LLC (Moscow, ID) established and licensed an innovative ELISA assay at the University of Idaho (Moscow, ID) for detection of serum PSPB in cattle, sheep, and goats after d 30 of gestation. This test has been commercially offered since 2003 under the registered trademark of BioPRYN®, which stands for Pregnant-Ruminant-Yes-No (Sasser et al., 2009). According to these authors, bioPRYN's ELISA assay is a typical sandwich ELISA developed using rabbit anti-PSPB antibodies raised against PSPB molecules isolated from placenta of cows before 100 d of gestation (as demonstrated by Butler et al. (1982). This assay exposes PSPB in maternal serum samples to the anti-PSPB antibodies that had been adsorbed onto 96-well microtiter plates. After binding to the wells, the PSPB/antibody complex is conjugated to horseradish peroxidase (HRP) allowing detection of PSPB and development of color. During the course of the assay, optical density (OD) from each well is estimated and compared to standard curve to obtain the concentration in ng/mL. A cutoff of > 0.5 ng/mL is normally used for determination of pregnancy on d 30 of gestation as described by Sasser (1986).

Gábor et al. (2007) examined the application of BioPRYN's ELISA assay to determine pregnancy and predict embryonic losses between d 30 and 36 of gestation. A total of 8118 serum samples were assayed from detection of PSPB from Hungarian dairy cows. Pregnancy was determined in cows with equal or greater average serum concentration of PSPB than the OD

cutoff equivalent to 0.024 ng/mL among triplicates. Embryonic loss was diagnosed by rectal palpation on d 60 after AI. Serum P4 was measured in cows determined as non-pregnant by bioPRYN's ELISA assay and in cows with serum PSPB between 0 and 30 % above the cutoff. Using this methodology, 50.3 % (4085/8118) of cows were determined pregnant. Among cows deemed pregnant by BioPRYN's assay, those with lower PSPB had greater chances of undergoing embryonic loss before d 60 of gestation. In fact, their findings suggest that most cows with low serum PSPB and < 2 ng/mL of serum P4 experienced embryonic loss before d 60 when both methods were used. These authors argue that using a percentage change of $\geq$ 20 % from a common baseline would favor detection of pregnancy and prediction of pregnancy losses using BioPRYN's ELISA assay.

Studies have demonstrated that comparing serum concentrations of PSPB within cow allows for accurate estimation of increase and diagnosis of pregnancy through BioPRYN's ELISA assay. Martins et al. (2018) studied the relationship between different concentrations of P4 during ovulatory follicle development on fertility parameters of lactating dairy cows. A within-cow baseline of serum PSPB was determined as the average concentration between d 16 and 20 post-AI. This baseline was used to estimate percentage increase between baseline and d 23, and cows with equal or greater than 28 % increase were considered pregnant. This method provided 98 % sensitivity and 97 % specificity which were numerically greater than when pregnancy was diagnosed with a single measurement of PSPB on d 23. These authors also demonstrated that a single sample of PSPB on d 28 can be highly accurate in determining pregnancies. They obtained 100 % sensitivity and 91.6 % specificity in determining pregnancy with a single measurement of serum PSPB using a cutoff of > 0.60 ng/mL to diagnose pregnancy on d 28 post-AI.

More recently, Middleton and Pursley (2019) investigated the accuracy of within-cow percentage change in serum PSPB between d 17 and 24 post-AI for identification of nonpregnant cows. These authors used a basal (d 17) serum PSPB measurements to calculate individual percentage change on d 24 in 206 synchronized and timed-AI lactating dairy cows. A $\geq$ 10 % change in serum PSPB from d 17 to 24 was used as the cutoff for determination of pregnancy. According to these authors, this cutoff represents the lowest percentage increase in serum PSPB among cows confirmed pregnant (102/206) in their study. Pregnancy diagnosis with transrectal ultrasonography on d 34 post-AI was considered the reference test. This method revealed 100 % sensitivity and 94 % specificity in identifying cows that were not pregnant on d 24 of gestation. These innovative approaches to the use of serum PSPB for diagnosis of pregnancy and prediction of pregnancy loss have greatly benefited the dairy industry. Identification of non-pregnant cows with maximum accuracy, as in the method previously described, allows for earlier resynchronization and AI, leading to less days to conception and greater productivity from dairy cows.

OTHER INDICATORS OF BOVINE CONCEPTUS DEVELOPMENT

In the last two decades, studies have shown a new method to indirectly assess bovine conceptus development during early pregnancy (Fricke et al., 2016; Carvalho et al., 2017; Pohler et al., 2017). This method involves observation of different patterns of relative expression of mRNAs for interferon-stimulated genes (ISGs) within peripheral blood leukocytes (PBL) as early as d 18 of gestation (Gifford et al., 2007; Green et al., 2010). The rationale behind this discovery is associated with changes induced by the production of IFNT by the developing conceptus (Bartol et al., 1985; Mann and Lamming, 2001). IFNT induces changes in the

endometrium and immunomodulatory pathways that lead to prolonged CL lifespan and tolerance to the semi-allogenic conceptus in the uterus. Consequently, IFNT produced by the conceptus alters gene expression in maternal leukocytes allowing for identification of different patterns of expression of ISGs between pregnant and nonpregnant dairy cows even before conceptus attachment (Garrett et al., 1988b; Robinson et al., 1999).

In a recent publication, Gifford et al. (2007) demonstrated differences in expression of ISGs in PBL of dairy cows on d 18 after AI. In their study, mRNA isolated from whole blood samples were subjected to RT-PCR for quantification of relative expression of ISGs [Mx-1, -2 (*Myxovirus*-resistant gene); ISG-15; IRF-1, -2 (interferon regulatory factor); and β2-MG (β2-microglobulin)]. Their findings demonstrated that Mx1, Mx2, and ISG-15 are activated in response to early pregnancy while IRF-1, IRF-2, and β2-MG are not. In fact, pregnant cows had greater relative expression of ISG-15 and Mx2 on d 18 after AI compared to nonpregnant cows. Similarly, Green et al. (2010) investigated potential candidates among several ISGs for early detection of pregnancy between d 15 and 18 of gestation with a microarray experiment. Increased expression of ISG-15 and Mx2 were observed in pregnant cows. However, classification data from their study (ROC curve) indicated there was a substantial number of false positives for pregnancy diagnosis with less accuracy within greater lactation cows. These authors argue that although this test shows favorable potential, more studies are necessary to identify the best approach (cutoff) when assessing bovine conceptus development.

THE RELATIONSHIP BETWEEN PROGESTERONE (P4) AND BOVINE CONCEPTUS DEVELOPMENT

These differences in fertility of lactating cows and heifers have been associated to variation in naturally occurring serum levels of P4 (Sartori et al., 2002, 2004). In their later publication (2004), these authors presented findings that revealed discrepancies in serum concentration of serum P4 between heifers and cows potentially associated with greater dry matter intake and metabolism of steroids in lactating cows. Their dataset also revealed that heifers had greater serum P4 concentration even though lactating cows had greater luteal volume. In a previous publication from the same group, lactating dairy cows had inferior embryonic development, fertilization rates, and embryo quality compared to nonlactating heifers (Sartori et al., 2002). At the time, these authors argued that such discrepancies in fertility of lactating cows and heifers were likely associated to the lower occurring concentration of P4 in lactating dairy cows during the early stages of pregnancy.

Several studies indicated that P4 induces changes in endometrial transcriptome leading to advanced conceptus development and greater synthesis of IFNT from the conceptus favoring progression of pregnancy (Mann et al., 2006; Carter et al., 2008; Forde et al., 2011). With a set of molecular studies using both *in vivo* and *in vitro* models, these authors revealed that P4 increases endometrial gene expression of genes responsible for synthesis and transport of nucleotides, amino acids, and energy sources towards the uterine lumen (Carter et al., 2008; Forde et al., 2009, 2011). The works of Garrett et al. (1988b), for example, demonstrated that treatment with exogenous P4 during the 1st 7 d of the estrous cycle increased content of polypeptides in uterine fluid between d 5 and 14 of gestation as well as length of conceptuses retrieved from treated cows on d 14. Carter et al. (2008) also showed that P4 supplementation on

d 3 of gestation with progesterone-releasing intravaginal devices (PRID) was associated with advanced conceptus elongation between d 13 and 16 of gestation.

Collectively, hatched embryos that were exposed to greater content of substrate within the histotroph had greater expansion and elongation. Clemente et al. (2009) presented data indicating that higher serum levels of P4 post-conception resulted in increased production of IFNT from concepti in both dairy cows and heifers. Mann et al. (2006) have argued that enhanced IFNT production from trophectoderm cells would lead to further endometrial transcriptome modifications that favor conceptus attachment, progression of pregnancy, and greater fertility. Hence, it appears that developing concepti may benefit from greater post-fertilization P4 whether endogenous or exogenously induced.

Strategies to supplement P4 post-fertilization

Substantial evidence on the effects of P4 on conceptus development and fertility parameters of dairy cows have supported the development of models to increase serum P4 post-fertilization. Predominantly, exogenous P4 strategies used intramuscular injections of P4 or intravaginal PRIDs (Garrett et al., 1988a; Carter et al., 2008). Endogenous strategies focused on the use of human Chorionic Gonadotropin (hCG) or GnRH to increase P4 from an original CL (Schmitt et al., 1996a) and/or to induce formation of accessory CL (Schmitt et al., 1996a; Santos et al., 2001).

As previously reviewed, both studies published by Garrett et al. (1988a) and Carter et al. (2008) are examples of exogenous supplementation of P4 during the early post-fertilization period. Garrett et al. (1988a) injected beef heifers with 100 mg of P4 intramuscularly on d 1, 2, 3, and 4 post-breeding. Carter et al. (2008), treated crossbreed heifers with a PRID on d 3 post-

estrus. Both methods were effective in increasing serum P4 from time of treatment through d 6 post-AI in treated heifers compared to controls.

Endogenous methods have primarily used hCG or GnRH to increase P4 from original CL and/or to induce formation of accessory CL (Schmitt et al., 1996a; Santos et al., 2001). Willard et al. (2003) evaluated the effect of treatment with GnRH on d 5 or 11 after AI on P4 concentration in heat-stressed lactating Holstein cows. In their study, injection of a GnRH agonist on d 5 or 11 resulted in ovulation of a first-wave follicle increasing CL volume and serum P4 on d 5 and 11 after AI. Garcia-Ispierto and López-Gatius (2012) presented similar outcomes after treating Spanish lactating Holstein cows with GnRH on d 5 post-AI. Their study revealed that treatment increased serum P4 between d 12 and 19 post-AI although only 1/10 had accessory CL induced by treatment. Another study, however, reported that treatment with GnRH on d 5 post-AI or its combination with GnRH on d of AI did not increase serum P4 on d 12 compared to untreated controls (Mendonça et al., 2017). This demonstrates some variability in outcomes of studies investigating the effect of GnRH post-AI on serum P4 of dairy cows.

Human chorionic gonadotropin is a glycoprotein normally produced by trophectoderm cells of developing human embryos. It has high similarity to luteinizing hormone (LH) which allows it to bind to LH receptors and exert LH-like effects in ruminants (Farin et al., 1988). When administered intramuscularly, hCG has long-lasting half-life in ruminants (24 to 33 h; Schmitt et al., 1996). Due to its LH-like actions, it is normally used to induce ovulation or to exert luteotropic effects on the developing CL. Human chorionic gonadotropin increased number and size of steroidogenic luteal cells leading to greater CL volume and higher serum P4 in ruminants (Farin et al., 1988). Schmitt et al. (1996) demonstrated that treatment with 3,000 IU of hCG on d 5 of the estrous cycle increased serum P4 between d 7 and 14 in nonlactating Holstein

cows compared to untreated controls and cows treated with GnRH on the same day. Similarly, Maillo et al. (2014) tested the effects of treatment with 3,000 IU of hCG on d 1, 2, 3, or 4 of the estrous cycle in crossbreed heifers compared to untreated controls. These authors confirmed that treatment with hCG as early as d 2 of the estrous cycle may result in greater serum P4 within 4 d after treatment. *Overall, a significant amount of data indicates that administration of hCG within 2 to 6 d after ovulation increases serum concentration of P4 in both heifers and lactating Holsteins and is* ***superior*** *to animals treated with GnRH around the same period.*

The potential paradox of P4 supplementation

O'Hara et al. (2014) observed a paradoxical effect of P4 supplementation on CL lifespan and conceptus development. In their study, crossbred nulliparous beef heifers received supplementation of P4 via PRID between d 3 and 5, d 3 and 7, or d 5 and 7 after detection of estrus and AI. Heifers were either slaughtered on d 14 or 16 of gestation. Data on CL size, length of conceptus, quantity of IFNT in uterine fluid, and serum P4 were collected. Their outcomes revealed that although supplementation of P4 had positive effects on length of conceptus and concentration of IFNT in uterine fluid, greater serum P4 may have induced short estrous cycles when supplemented during early metestrus.

Such detrimental effects have been associated with early decline in P4 shortly after the effects of treatment are ceased. Garrett et al. (1988a) demonstrated that supplementation with exogenous P4 via intramuscular injection on d 1, 2, 3, and 4 of the estrous cycle may stimulate early pulsatile release of $PGF_{2\alpha}$ from the endometrium. As a consequence, supplementation of exogenous P4 may lead to reduction of CL lifespan, shortening of interestrus intervals, and detrimental effects to fertility in cows and heifers. These findings indicate that strategies to

increase serum P4 may demand the utilization of endogenous/indirect routes to avoid unfavorable effects on pregnancy.

POST-OVULATION hCG TREATMENT AS AN STRATEGY TO INDUCE THE FORMATION OF ACCESSORY CORPORA LUTEA (CL) AND IMPROVE FERTILITY OF DAIRY COWS

It is unclear whether P4 supplementation via post-ovulation hCG treatment translate into favorable outcomes of pregnancies per AI (P/AI) and pregnancy loss. Studies have demonstrated inconsistent results when investigating the effects of P4 supplementation on fertility of dairy cows. A summary of available P/AI data is presented in table 1.1. Studies were selected based on 3 main criteria: (1) sample size ($n \geq 150$ cows), (2) dose ($\geq$ 3,000 IU of hCG), and (3) d of treatment with hCG (between d 4 and 9 post-ovulation).

Potential reasons for inconsistency of outcomes in fertility of dairy cows treated with hCG post-ovulation are (1) disparities in body condition score (BCS) within study subjects, (2) differences in weather conditions among studies, (3) lack of randomization within parities, (4) negative effects of higher serum P4 post-ovulation on CL lifespan, (5) development of humoral response against hCG preparations following treatment, (6) differences in prevalence of pregnancy loss among herds, or (7) side of formation of accessory CL.

Changes in BCS are known to affect fertility of dairy cows inseminated after timed-AI programs (Moreira et al., 2000; Middleton et al., 2019). Among the studies presented in Table 1.1, only one (Santos et al., 2001) investigated the influence of BCS on fertility of cows treated with hCG post-AI. In that study, cows with BCS greater than 2.75 (in a 1 to 5 scale) on the d of AI had higher conception rates than those with equal or lower BCS. A positive effect of hCG

was observed among cows that lost BCS during the study. Amongst cows that lost BCS, those treated with hCG had greater conception rate on d 28 than untreated controls. Therefore, it is likely that divergence in outcomes between studies investigating post-ovulation hCG treatment may be explained by variation in BCS between cows.

Weather conditions can also significantly affect fertility of dairy cows. Sartori et al. (2002) reported lower fertility of lactating dairy cows in warmer compared to colder seasons. This has been attributed to lower quality of oocytes and embryos triggered by heat stress. Studies have hypothesized that post-ovulation treatment with hCG would increase serum P4 and support embryonic development as a potential strategy to overcome such effect on fertility. However, their findings have also been inconsistent. Among those described in Table 1.1, only two studies reported data on interactions between climate and effect of treatment with hCG. Santos et al. (2001a) reported greater conception rate in hCG-treated cows during the cool season, but no difference was identified during the warm season. Inversely, Shabankareh et al. (2010) observed increased P/AI in hCG-treated cows compared to untreated controls during summer. Thus, it seems unclear whether post-ovulation hCG treatment is an adequate strategy to overcome fertility issues associated with heat-stress.

The effect of parity on fertility is undisputed. A significant amount of data demonstrates that decreased fertility is associated with greater parity in dairy cows (Pursley et al., 1997; Gröhn and Rajala-Schultz, 2000; Minela et al., 2021). Nulliparous heifers and primiparous cows have greater fertility than multiparous cows. This effect has been predominantly linked to consequences of greater milk production on increased dry matter intake and steroid metabolism (Sartori et al., 2002, 2004). Therefore, studies on the effect of treatment with hCG post-ovulation on fertility require assessment of effect of treatment within parities. Different approaches to the

application of parity in randomization and study designs may have led to divergent outcomes among studies. For instance, Schmitt et al. (1996b) designed two different experiments to test the effect of treatment with hCG on nulliparous heifers and multiparous cows. Treatment with hCG did not affect conception rates in both nulliparous and multiparous cows although no comparison between heifers and cows was possible. Yet, both Shabankareh et al., (2010) and Nascimento et al. (2013) reported increased conception rates among primiparous cows treated with hCG, but no differences among multiparous cows. In parallel, Stevenson et al. (2007) demonstrated an increment of 2.6 percentage points in the conception rate of second parity cows treated with hCG in comparison to untreated controls. However, no increment was observed among primiparous or $\geq 3^{rd}$ parity cows. Santos et al. (2001a), on the other hand, used a different approach when correcting for the potential confounding effect of parity on fertility. These authors grouped cows by quartiles of milk production and tested for potential interaction with effect of treatment. Although greater milk production was associated with decreased fertility, no interaction between milk production and treatment was identified.

As previously reviewed, O'Hara et al. (2014) demonstrated a negative effect of P4 supplementation on CL lifespan and conceptus development. Although supplementation of P4 had positive effects on size and levels of IFNT being produced by the conceptus in their study, it reduced length of estrus cycle and CL lifespan when supplemented to crossbreed nulliparous beef heifers during early metestrus. Similarly, Garrett et al. (1988a) had previously demonstrated that P4 supplementation via intramuscular injection on d 1, 2, 3, and 4 of the estrous cycle stimulates early pulsatile release of $PGF_{2\alpha}$ from the endometrium leading to reduction of CL lifespan and shortening of interestrus intervals. Thus, it is likely that some variation in serum

concentration of P4 due to post-ovulation hCG treatment may explain the inconsistency in fertility outcomes of lactating dairy cows.

Another potential explanation for inconsistent results of post-ovulation hCG treatment is the development of humoral immune response against hCG molecules in cows. Giordano et al. (2012) reported that some cows developed antibodies against hCG following repeated treatment. According to their findings, levels of antibodies against hCG increased from d 0 after treatment to maximum levels on d 14, decreasing progressively thereafter. Although it is still unclear if these antibodies are capable of blocking hCG from binding to LH receptors, variation in immune response between herds could explain discrepancies in fertility outcomes following hCG treatment.

Among the literature presented in table 1.1, only two studies have reported data on prevalence of pregnancy loss after post-ovulation treatment with hCG. Santos et al. (2001) reported no statistical difference ($P > 0.20$) in pregnancy loss between d 28, 45, or 90 post-AI when comparing hCG-treated cows to untreated controls. Although only numerically different, 19 % of untreated cows vs. 16.1 % of cows treated hCG experienced pregnancy loss between d 28 and 90 post-AI. Stevenson et al. (2007) evaluated the effect of hCG treatment between d 4 and 9 post-AI on pregnancy survival in 5 dairy herds. These authors reported a tendency ($P = 0.09$) for treatment with hCG to reduce pregnancy survival among all herds in which 97.3 % of untreated cows and 95.7 % of cows treated with hCG maintained pregnancy after the first pregnancy diagnosis. Consequently, the hypothesis that post-ovulation hCG treatment would improve fertility by decreasing pregnancy loss also lacks evidence.

Monteiro et al. (2021) developed a study to investigate differences in time of luteolysis of accessory CL during pregnancy of dairy cows. Although their study used GnRH as a method of

inducing accessory CL, their dataset demonstrated differences in time and rate of luteolysis of accessory CL by side. The majority of contralateral accessory CL (88.9 %) underwent luteolysis during the 1st and 2nd month of pregnancy while no ipsilateral accessory CL had luteolysis. These authors speculate that the same local mechanisms that prevent regression of the original CL would also prevent the regression of ipsilateral accessory CL. Conceivably, some uncertainty on the effects of treatment with hCG post-ovulation on fertility of dairy cows may be related to the side of ovulation after treatment with hCG.

Post-ovulation treatment with hCG could increase milk production costs by extending time to conception and reducing fertility in subsequent lactations of treated dairy cows. Cunha et al. (2021) demonstrated that treatment with hCG between d 5 and 7 of the estrous cycle increased length of the estrous cycle of those cows that returned to estrus. That is, cows that do not become pregnant at the 1st AI followed by hCG treatment are more likely to have longer intervals between services and days to conception. Therefore, these cows are more inclined to exceed a threshold of 130 days in milk (DIM) until conception. Middleton et al. (2019) identified that cows that conceive after 130 DIM have higher risk of health issues, less chances of conceiving, and greater chances of undergoing pregnancy loss in the next lactation. Therefore, treatment with hCG has the potential to extend time to conception within one lactation and disrupt conception, health, and pregnancy in the subsequent lactations.

In summary, while treatment with hCG post-ovulation is an excellent strategy to increase serum P4 after fertilization in experimental settings, the ideal application of this strategy in commercial dairy farms is yet to be recognized. Although some studies report significant increases in P/AI as an effect of treatment, these outcomes generally seem to only be repeatable in primiparous cows. The experiment presented in Chapter 2 and 3 of this thesis aims to study

the effects of treatment with hCG post-ovulation on time to conceptus attachment when associated to Double-Ovsynch and Ovsynch protocols in lactating dairy cows.

KEY ASPECTS OF PREGNANCY LOSSES IN LACTATING DAIRY COWS

Pregnancy losses among lactating dairy cows represent an alarming portion of expenses for dairy producers (Martins et al., 2018). In addition to the costs of labor, drugs, and semen, pregnancy losses also prolong DIM until successful pregnancy and increase chances of premature culling. Advancements on fertility programs in the last decades have improved overall fertility of lactating dairy cows by reducing the occurrence of double ovulations and twining, and improving luteolysis (Bello et al., 2006; Brusveen et al., 2009; Wiltbank and Pursley, 2014; Martins et al., 2018). Still, significant discrepancies exist between pregnancies per AI (P/AI) and calving rates.

Twining is one of the major contributors for overall pregnancy loss in dairy cattle. Echternkamp et al. (2007) designed a study to investigate the effects of number and distribution of conceptuses on gestation length, occurrence of dystocia, sex ratio, survival, and performance of single, twin, and triplet calves. Cows were phenotypically selected for double ovulation and twinning. Their results indicated that twinning increased the chances of late pregnancy loss and dystocia while producing performance of calves that survived.

Sartori et al. (2002) have reported high fertilization rates (87.8 %) in high producing dairy cows. They have observed that high rates of early embryonic mortality with greater occurrence of low-quality grade and abnormal embryos in high producing dairy cows were associated. Even though their study was not designed to identify the direct causes for these

outcomes, these authors speculated that poor oocyte quality may be compromising embryo quality and increasing early pregnancy losses in high producing cows.

Martins et al. (2018) studied the effect of different levels of serum P4 during ovulatory follicle development on pregnancy loss in lactating dairy cows. They were able to diagnose pregnancy as early as d 23 post-AI and revealed P/AI on d 23 ranging from 48.1 to 66.4 % among treatment groups. Pregnancy losses between d 23 and 56 post AI that ranged from 3.6 to 12.4 %, with greater percentage of double ovulation and pregnancy losses among cows with low P4 during follicular development. Birth of live calves among all inseminated cows (calving rates) ranged from 36.1 to 49.5 %. These authors discussed that the occurrence of such expressive discrepancy between pregnancy rates on d 23 post-AI and calving rates are associated with low P4 during ovulatory follicle development. That is, low P4 during ovulatory follicle development enables the occurrence of double ovulation and twinning leading to overcrowding, placentation issues, and pregnancy loss. Their estimation, provided with high accuracy using differences in serum PSPB, offered a unique perspective of pregnancy loss and the potential association with serum P4 during follicular development.

In a study by Parr et al. (2012), the relationship between P/AI and post-fertilization serum P4 was investigated in nulliparous dairy heifers. Measurement of serum P4 on d 4, 5, 6, and 7 after detection of estrus allowed identification of a positive linear and quadratic relationship between levels of P4 and P/AI were observed. Their data indicated that P/AI increased with greater serum P4 post-fertilization. However, P/AI decreased as serum P4 exceeded optimum concentrations of 2.5, 4.0, 5.0, and 5.2 ng/mL on d 4, 5, 6, and 7, respectively.

Altogether, it becomes evident that levels of serum P4 prior and after fertilization were positively associated with embryonic development and fertility parameters in dairy cows. Yet, no

study has investigated the effects of P4 supplementation during early pregnancy on time to conceptus attachment in dairy cows. In Chapter 2 and 3, we propose an experiment to test the effects of treatment with hCG on d2, d5, and d2 and 5 post-ovulation on luteal function in lactating Holstein cows, and to assess the relationship between post-ovulation luteal function and time to conceptus attachment as a function of temporal changes in PSPB concentration. In Chapter 4, we summarize the findings of our experiment and discuss the financial implication of using these strategies to improve fertility on dairy farms.

APPENDIX

Table 1.1. Summary of outcomes of studies investigating the effect of treatment with ≥ 3,000 IU of hCG post-AI on pregnancies per AI (P/AI) between d 28 and 60 after AI in lactating dairy cows by parity.

Authors	Day of treatment[1]	Fertility program[2]	P/AI [% (no./total)]			
			Control	hCG	*Effect (%)*	*P-value*[3]
Primiparous						
Shams-Esfandabadi et al. (2007)	d 5	NE	22.7 (5/22)	20 (2/10)	-2.7	0.54
Stevenson et al. (2007)	d 4 - 9	ES/Pre-Ov	32.8 (246/750)	33.2 (250/753)	+0.4	>0.05
Shabankareh et al. (2010)	d 5	Ov/EB-FTAI/NE	34.9 (51/146)	57.9 (81/140)	+23.0	<0.01
Nascimento et al. (2013)	d 5	DO/Pre-Ov	39.5 (215/544)	49.7 (266/535)	+10.2	<0.01
Overall			35.4 (517/1462)	41.7 (599/1438)	+6.3	-
Multiparous						
Schmitt et al. (1996b)	d 5 - 6	ES	23.5 (24/102)	24.2 (24/99)	+0.7	0.91
Shams-Esfandabadi et al. (2007)	d 5	NE	33.3 (22/66)	28.3 (17/60)	-5.0	0.86
Stevenson et al. (2007)	d 4 - 9	ES/Pre-Ov	26.2 (462/1759)	33.0 (464/1405)	+6.8	<0.01
Shabankareh et al. (2010)	d 5	Ov/EB-FTAI/NE	26.6 (51/192)	32.3 (61/189)	+5.7	0.22
Nascimento et al. (2013)	d 5	DO/Pre-Ov	36 (351/975)	35.7 (330/925)	-0.3	0.98
Overall			29.4 (910/3094)	33.5 (896/2678)	+4.0	-
Unspecified Parity						
Santos et al. (2001a)	d 5	ES	38.7 (79/203)	45.8 (93/203)	+7.1	0.01
Total			31.6 (1506/4759)	36.8 (1588/4319)	+5.1	-

[1]Day of treatment relative to d of AI.
[2]Fertility program used in the study to determine time of AI before treatment: "natural estrus" (NE), "estrus synchronization" (ES), "Ovsynch" (Ov), "Presynch-Ovsynch" (Pre-Ov), "Estradiol-based fixed-time AI" (EB-FTAI), and "Double-Ovsynch" (DO).
[3]Presented *P*-value for difference between treatment with hCG and control based on Chi-square tests.

CHAPTER 2

EFFECT OF INDUCING ACCESSORY CL DURING EARLY EMBRYONIC DEVELOPMENT ON TIME TO CONCEPTUS ATTACHMENT IN LACTATING DAIRY COWS: PART I. IMPACT OF hCG ON OVARIAN FUNCTION

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INTRODUCTION

Understanding models to increase circulating progesterone (P4) post-ovulation is critical for advancing research on reproductive management of dairy cattle. Substantial evidence has linked greater post-fertilization ovarian function and serum P4 to enhanced conceptus development and fertility parameters in ruminants (Carter et al., 2008; Clemente et al., 2009; Forde et al., 2009). This has supported the development of models to increase serum P4 post-ovulation. Endogenous strategies focused on the use of human Chorionic Gonadotropin (hCG) or GnRH to increase P4 from an original CL (Maillo et al., 2014) and/or to induce formation of accessory CL (Santos et al., 2001; Nascimento et al., 2013)

In cattle, human chorionic gonadotropin (hCG) binds to LH receptors and elicits LH-like effects for longer period of time than endogenous LH surge induced by a GnRH-agonist (Schmitt et al., 1996b). It has proven effective in increasing endogenous levels of P4 post-ovulation through the formation of accessory CL (Santos et al., 2001) and/or stimulating the production of P4 by small luteal cells of developing CL (Maillo et al., 2014). Several studies have demonstrated greater CL volume associated with increase in serum P4 between d 7 and 14 when beef-cross heifers (Rizos et al., 2012; Maillo et al., 2014), dairy heifers (Diaz et al., 1998), or lactating cows (Stevenson et al., 2007; Nascimento et al., 2013) were treated with $\geq$ 2,000 IU of hCG on d 5 of the estrous cycle.

Studies suggested that hCG is more effective than GnRH in inducing ovulation of a first-wave dominant follicle and increasing serum P4 early in the estrous cycle. Schmitt et al. (1996a) demonstrated that 3,000 IU of hCG on d 5 induced larger accessory CL and higher concentration of P4 than treatment with GnRH on the same day in nulliparous Holstein heifers. Similar outcomes have also been demonstrated in lactating dairy cows in which treatment with hCG

between d 4 and 9 post-ovulation induced greater accessory CL and change in serum P4 compared to both GnRH and P4-releasing intravaginal device (PRID) (Stevenson et al., 2007). More recently, Cabrera et al. (2021) confirmed that treatment with ≥ 2,500 IU of hCG on d 7 of the estrous cycle of lactating Holstein cows resulted in greater ovulatory response, combined CL volume, and circulating P4 than GnRH.

We hypothesized that treatment with hCG during early diestrus following AI would increase luteal volume during the pre-attachment phase of embryonic development in lactating dairy cows. Therefore, this study aimed to investigate the effects of treatment with hCG on d2, d5, and d2 and 5 post-ovulation on luteal function of lactating Holstein cows. Outcomes of this experiment were then used in Part II that aimed to investigate the relationship between luteal function and time of conceptus attachment (CA) in lactating dairy cows.

MATERIALS AND METHODS

Cows and Housing

This experiment was conducted at Nobis Dairy Farms in St. Johns, MI, between January and April of 2020. The herd consisted of approximately 1,100 Holstein cows milked 3-times a day with rolling herd average 13,971 kg of milk. Cows were housed in ventilated freestall barns with grooved concrete floors, head locks, and free access to food and water. A TMR was fed once daily and consisted of alfalfa and corn silage and concentrates meeting or exceeding nutrient requirements for lactating dairy cows (NRC, 2001).

All procedures in this experiment were performed by trained students and laboratory personnel and approved by the Institutional Animal Care & Use Committee at Michigan State University prior to execution.

Experimental Design

As Part I of a two-part study, outcomes of this trial were analyzed within a sample size calculated based on a power analysis for the main outcome of interest, time to conceptus attachment. Power analysis indicated that 90 cows per treatment would be sufficient to detect a difference of 2 d to conceptus attachment with a standard deviation of $\pm$ 1 d to minimize a type II error $\leq$ 20 % at a significance level of $\leq$ 0.05. Weekly cohorts of lactating Holstein cows (n = 368) were synchronized with Double-Ovsynch (1st service; Souza et al., 2008) or resynchronized with Ovsynch (2nd service or greater; Brusveen et al., 2008) prior to enrollment in the study. The initial GnRH (86 μg gonadorelin acetate; Fertagyl®, Merck Animal Health, Kenilworth, NJ) of Double Ovsynch started at 48 $\pm$ 3 DIM. Timed-AI was performed in all cows 16 h after the last GnRH of Double Ovsynch (1st service; 77 $\pm$ 3 DIM) or Ovsynch in 2nd+ service cows that were resynchronized. All $PGF_{2\alpha}$ administrations utilized 500 μg cloprostenol sodium (Estrumate®, Merck Animal Health, Kenilworth, NJ).

As a reference, day of ovulation was considered the most critical point of measurement for calculation of time of fertilization due to the short lifespan of the oocyte (Szollosi, 1973; Hunter, 1985). Day of ovulation in this study was calculated as 24 to 32 h (Pursley et al., 1995) after final GnRH of Double Ovsynch or Ovsynch. All outcomes in this study are related to days post-ovulation.

Cows with functional CL and at least one large ($\geq$ 10 mm) follicle on the day of $PGF_{2\alpha}$ 56 hours prior to final GnRH of Double Ovsynch or Ovsynch were considered synchronized. CL function was determined by the presence of blood flow through the entire tissue of each CL via color Doppler. Only synchronized cows were randomized in blocks by service and parity and assigned into one of four treatments: 3,000 IU of hCG (Chorulon®, Merck Animal Health,

Kenilworth, NJ) on day 2 post-ovulation (D2), 3,000 IU of hCG on day 2 and 5 post-ovulation (D2&5), 3,000 IU of hCG on day 5 post-ovulation (D5), and no treatment (Controls; Figure 2.5).

Administrations of $PGF_{2\alpha}$, GnRH, and hCG were performed in either the semitendinosus or semimembranosus muscles using 3.5-cm, 20-gauge, single-use needles, and 3-mL, single-use syringes (BD, Franklin Lakes, NJ, USA).

Ovaries of all cows were evaluated and mapped via transrectal ultrasonography using a color Doppler MyLab™ GammaVET equipped with a 5-10 MHz multifrequency linear array probe (Esaote North America Inc., Indianapolis, IN). Follicle and CL diameters (D) were determined as the average of the cross section at its largest diameter using built-in calipers. Small follicles (≤7 mm) had their sizes estimated by built-in lateral grids on the machine's monitor, and only follicles with average diameter ≥ 3 mm were recorded. Ovaries of all cows were scanned and mapped on d 0, 5, and 10 post-ovulation. Synchronization of ovulation, and ovulation in response to treatments, were determined by the disappearance of the ovulatory follicle and presence of newly formed CL on d 5 and 10 post-ovulation. Fluid-filled CL cavities were measured and subtracted from the final calculation of CL diameter. CL volume was estimated as $V= 4/3 \times \pi \times R^3$, where radius (R) was calculated as $R = D/2$. Pregnancy diagnosis on d 35, 63, or 100 post-AI/ovulation was performed by the farm's veterinarian through transrectal ultrasonography.

Blood samples were collected from all cows for analysis of progesterone (P4). Samples were obtained on days 0, 5, 19 and 20 post-ovulation. Whole blood was collected via coccygeal vena puncture into vacuum tubes with clot activator and gel for serum separation (BD Diagnostics, Franklin Lakes, NJ). Samples were immediately placed in ice, transported to the laboratory, and incubated at 4° C for 24 h. All samples were centrifuged for 20 min at 2,016 x *g*

at 4° C (Sorvall™ ST 40R, Thermo Fisher Scientific Inc., Waltham, MA). Aliquots of serum were transferred into 1.5 mL, labeled microtubes, and frozen at -20° C until assayed.

All frozen serum samples were shipped overnight to bioPRYN, bioTRACKING LLC, Moscow, ID where they were analyzed. P4 concentrations were determined by an enzyme immunoassay (EIA) developed bioTRACKING LLC. Briefly, 96-well Costar Stripwell plates (Corning Inc. Corning, NY) were coated with a BSA-P4 conjugate (Product #2606, Steraloids, Inc., Newport, RI). A mouse monoclonal antibody to P4 (Product #MAB286P, Maine Biotechnology Services, Portland, ME) was labeled with biotin according to the manufacturer's instructions (EZ-Link™ Sulfo-NHS-LC-Biotin, Thermo Fisher Scientific, Rockford, IL). For the assay, a standard curve of known progesterone concentrations in PBS (phosphate buffer saline pH 7.4) was run in duplicate along with serum samples. Twenty-five µL of each standard and serum sample were added to each well of the coated microplate. Immediately 25 µL of biotin labeled anti-P4 antibody at a concentration of 100 ng/mL in PBS was added to each well and mixed. The assay was incubated for 30 min at room temperature. Plates were then washed three times with PBS. Fifty µL of streptavidin-peroxidase conjugate (#S911, Thermo Fisher Scientific, Eugene, OR) was added to each well and incubated for 30 min at room temperature. After incubation, plates were wash 3 times with PBS and 50 µL of 3,3',5,5',-Tetramethylbenzidine was added to each well and incubated for other 15 min at room temperature. A fluoride stop-solution (50 µL) and was added to the reaction and optical density for each well was obtained from a plate reader (BioTek 800TS, BioTek Instruments, Inc. Winooski, VT) with a filter wavelength of 650 nm. Concentrations of samples were determined using a 4-parameter fit using the Gen5 3.03.14 program (BioTek Instruments, Inc. Winooski, VT). The lowest level of P4 detectable in this assay was 0.045 ng/mL. A control sample was run in duplicate (1.5 ng/mL of progesterone

in PBS) on each assay plate to measure the precision of the assay. Overall intra- and inter-assay CV across 42 plates were 5.03 and 9.36 %.

Data Management, Sample Size, and Statistical Analysis

Herd information such as weekly cohort, DIM, allocation group (pen), service, and parity were retrieved from the farm's data management software (PCDART, Dairy Records Management Systems, Raleigh, NC). All datapoints were entered weekly into one single Excel spreadsheet (Microsoft Corp., Redmond, WA).

Binomial outcomes were analyzed through logistic regression models using the LOGISTIC procedure in SAS (9.4, SAS Institute Inc., Cary, NC) under the LOGIT function. Final models considered the effect of treatments (D2, D2&5, D5, and Controls), service (1^{st} service or resynchronization), parity (primiparous or multiparous), number of ovulations to the GnRH of the synchronization protocol (single or double), occurrence of accessory CL relative to the original CL (at least one or none), side of accessory CL relative to the original CL (ipsilateral or contralateral), and pregnancy status on d 35 post-ovulation (pregnant or non-pregnant). Two-way interactions were tested among all variables considering a hierarchical approach. Interaction terms and variables were only maintained in the final models if $P < 0.20$. Frequency and proportions were obtained using the FREQ procedure in SAS and tested by Chi-square test of homogeneity.

Continuous outcomes were analyzed through linear mixed models using the MIXED procedure in SAS. Linear models examined the same fixed effects and two-way interactions as those in the logistic models also following a hierarchical approach. Interaction terms and variables were only upheld in the final linear models if $P < 0.20$. All reported continuous means were obtained using PROC MEANS procedure in SAS.

Analysis for effect of treatment on occurrence of accessory CL (binomial; "≥ 1" or "none") and volume of original CL (continuous; "mm^3") used one-tailed tests for superiority of outcome in treated groups compared to controls. All other analyses applied 2-tailed tests. Effect of treatment on volume of original CL and serum P4 on d 19 and 20 post-ovulation were compared to controls using pre-established orthogonal contrast (Control vs. D2; Control vs. D2&5; Control vs. D5). Tukey's adjustment was applied to all predetermined pairwise comparisons with > 2 covariates. Significant differences were considered when *P* values were ≤ 0.05 and tendencies were reported when *P* values were > 0.05 and ≤ 0.10. Normality assumptions were determined by visual examination of datasets and though testing with Shapiro Wilk's test using UNIVARIATE procedure in SAS (normal distribution as the null hypothesis).

RESULTS

Effect of treatment on number and side of accessory CL

The final GnRH of Double Ovsynch or Ovsynch induced double ovulations in 11.7 % (43/368) of the cows in this study. The distribution of double ovulations was homogeneous across treatments (Controls, 10 % - 9/92; D2, 12 % - 11/92; D2&5, 11 % - 10/92; D5, 14 % - 13/92; $P = 0.8$).

Newly formed accessory CL on d 10 post-ovulation induced in response to treatment with hCG were greater in treatments D2&5 and D5 compared to D2 (24.7 % - 20/81) and controls (Table 2.1). There was a tendency for greater accessory CL in primiparous cows than multiparous. However, among treated cows, a greater ($P < 0.01$) percentage of primiparous cows (80.3 % - 53/66) had accessory CL than multiparous cows (64.2 % - 113/176). Yet, no interaction was observed between treatment and parity ($P = 0.69$).

Of all cows with accessory CL, 43.4 % (72/166) had accessory CL ipsilateral to the single ovulation induced by the final GnRH. No difference was observed in the distribution of accessory CL by side among treatments. A greater percentage of primiparous cows had ipsilateral accessory CL than multiparous cows (Table 2.1).

Newly formed accessory CL were observed on d 5 post-ovulation in response to hCG on d 2 post-ovulation in 17 % of cows in treatment D2 (16/92) and 9 % in treatment D2&5 (9/92). Among those in treatment D2&5 that ovulated between d 2 and 5 post-ovulation, 8 of 9 had a second accessory CL induced between d 5 and d 10 post-ovulation. Cows with double-ovulation and newly formed CL in response to hCG on d 2 were removed from analysis of CL volume to avoid variability due to unprecise measurement of CL volume at the time of ultrasound examination.

Effect of treatment on volume of original CL

There was clear homology of ovulatory follicle (OF) diameter at time of final GnRH of Double Ovsynch and Ovsynch (Controls, 16.32 $\pm$ 0.2; D2, 16.54 $\pm$ 0.23; D2&5, 16.64 $\pm$ 0.21; D5, 16.35 $\pm$ 0.25 mm; $P = 0.7$).

Treatment with hCG increased volume of the original CL on d 10 post-ovulation in treatments D2 (12,190.40 $\pm$ 460.29 mm^3), D2&5 (14,648.50 $\pm$ 607.84 mm^3) and D5 (13,181.80 $\pm$ 536.24 mm^3) compared to controls (10,843.39 $\pm$ 479.35 mm^3; Figure 2.1). Parity did not alter ($P = 0.17$) luteal volume of the original CL at d 10. There was no interaction between treatment and service, treatment and parity, or treatment and number of ovulations to the last GnRH ($P > 0.25$).

When cows with double ovulation and those with ovulation induced by hCG on d 2 post-ovulation were removed from the analysis, treatments D2 (11,882.02 $\pm$ 512.44 mm^3), D2&5 (13,583.13 $\pm$ 590.63 mm^3), and D5 (12,440.30 $\pm$ 508.34 mm^3) had greater increased volume of

the original CL compared to controls (10,298.00 ± 456.05 mm^3; Figure 2.1). No effect of parity ($P = 0.51$) or interaction between treatment and parity was observed ($P > 0.25$).

Effect of treatment on combined CL volume of original and new accessory CL

Treatment with hCG induced greater combined CL volume on d 10 (total CL volume in both ovaries at the time of ultrasound examination) post-ovulation compared to controls (10,843.39 ± 479.35 mm^3). Treatment D2&5 (19,437.20 ± 713.2 mm^3) had the greatest combined CL volume followed by treatments D5 (16,914.45 ± 609.07 mm^3) and D2 (13,295.17 ± 499.41 mm^3; Figure 2.2). As expected, cows with double ovulation (20,587.51 ± 1118.79 mm^3) had greater ($P < 0.001$) combined CL volume on d 10 post-ovulation than those with single ovulation (14,399.5 ± 332.78 mm^3). However, no direct effect of parity ($P = 0.49$) or interaction between treatment, number of ovulations to the last GnRH, parity, or service were observed ($P > 0.25$).

When cows with double ovulation and those with induced ovulation to hCG on d 2 post-ovulation (24/368) were removed from the analysis, treatment D2&5 (18,008.96 ± 705.92 mm^3) and D5 (16,173.25 ± 588.61 mm^3) had greater combined CL volume on d 10 post-ovulation compared to controls (10,298.00 ± 456.05 mm^3) and D2 (12,158.97 ± 506.27 mm^3; Figure 2.2). A tendency for difference between D2&5 and D5 as well as D2 and Control was observed. No difference ($P = 0.7$) was observed between primiparous (13,891.22 ± 703.95 mm^3) and multiparous cows (14,197.54 ± 381.12 mm^3).

Effect of treatment on serum P4

Treatment with hCG on d 2 increased serum P4 in d 5 post-ovulation in the absence of accessory CL induced by hCG on d 2 (Figure 2.4). No difference was observed in serum P4 within d 19 and 20 ($P = 0.29$). Therefore, average serum P4 between d 19 and 20 was used as

response variable to reduce potential day-to-day variability during the analysis when determining effect of treatment. Treatment D2&5 and D5 induced greater average serum P4 on d 19 and 20 post-AI/ovulation compared with D2 or controls. Serum P4 averaged (± SEM) 6.13 ± 0.31 ng/mL in D5, 6.10 ± 0.24 ng/mL in D2&5, 4.80 ± 0.28 ng/mL in D2, and 4.19 ± 0.22 ng/mL in controls (Figure 2.3). No interactions were observed between treatment and number of ovulations ($P = 0.92$), nor between treatment and parity ($P = 0.24$).

There was an effect of treatment across pregnant cows with single ovulation or no induced ovulation to treatment with hCG on d 2 post-AI (146/368). Treatments D2&5 (6.03 ± 0.26 ng/mL) and D5 (6.10 ± 0.33 ng/mL) increased average serum P4 between d 19 and 20 post-AI compared to treatment D2 (4.67 ± 0.31 ng/mL) and controls (4.09 ± 0.21 ng/mL; Figure 2.3). Yet, no effect of treatment was observed among cows with double ovulation (43/368; $P = 0.65$). No interaction was observed between treatment and parity ($P = 0.47$). And no effect of side of accessory CL was observed ($P = 0.59$).

DISCUSSION

Treatment with 3,000 IU of hCG on d 2 and 5 or a single treatment with hCG on d 5 post-ovulation was highly effective in inducing ovulation of first-wave dominant follicles, developing accessory CL, increasing CL volume, and serum P4 in lactating Holstein cows. A single treatment of hCG on d 2 also increased volume of the original developing CL on d 10 and increased serum P4 on d 5 post-ovulation. Therefore, this study confirmed our hypothesis that treatment with hCG during early estrous cycle would increase CL number and volume and induce greater serum P4 during the pre-attachment phase of embryonic development in lactating Holstein cows.

Past studies demonstrated similar outcomes when investigating the effects of post-ovulation treatment with hCG in cattle. Nascimento et al. (2013) presented that nearly 80 % of cows treated with 2,000 IU of hCG on d 5 post-ovulation ovulated one or more follicles and had greater serum P4 by d 12 post-AI. Other studies have reported similar findings in which > 81 % of lactating cows ovulated in response to treatment with ≥ 3,000 IU of hCG between d 4 and 9 of the estrous cycle (Santos et al., 2001; Stevenson et al., 2007; Cabrera et al., 2021). In the present study, ovulation was observed in the vast majority of cows treated with hCG on d 5 post-ovulation (89 % in D2&5, and 92.4 % in D5). Despite ovulation rates being greater in treatments D2&5 and D5 than in treatment D2 and controls (Table 2.1), ovulation was also observed after treatment on d 2. Unexpectedly, we observed 17 % of cows in treatment D2 (16/92) and 9 % in treatment D2&5 (9/92) had new ovulations / accessory CL on d 5 post-ovulation (3 d after treatment). We speculate that the long half-life of hCG (24 to 33 h; Schmitt et al., 1996) caused ovulation of some cows as soon as deviation of the dominant follicle from subordinates. This would have been approximately 2 d after treatment with hCG on d2 of the estrous cycle. Also, unexpectedly, 8 of these 9 cows in D2&5 ovulated in response to the D5 treatment.

There was a tendency for primiparous cows to have greater ovulatory response than multiparous among all cows enrolled in the study. The lack of sufficient evidence for difference between parities was likely associated to the absence of ovulation among untreated controls. When controls were removed from the analysis, a greater percentage of primiparous cows had accessory CL in response to treatment than multiparous cows. However, the effect of treatment within parity did not differ. These outcomes diverge from previous studies that demonstrated nonexistent relationship of parity with ovulatory response to treatment with hCG despite

significant interaction between treatment and parity on serum P4 after treatment (Santos et al., 2001; Cabrera et al., 2021).

Monteiro et al. (2021) demonstrated differences in time and rate of luteolysis of accessory CL by side relative to the original CL. In their study, the majority of contralateral accessory CL (88.9 %) underwent luteolysis during the first trimester of pregnancy while no ipsilateral accessory CL had luteolysis. These authors speculate that the same local mechanisms that prevent regression of the original CL would also prevent the regression of ipsilateral accessory CL. Other reports indicate that side of ovulation induced by post-ovulation hCG treatment is inconsistent and unpredictable. Santos et al. (2001a), for example, reported that a greater percentage of treated cows had contralateral accessory CL than ipsilateral CL in their study. Other findings by Baez et al. (2017) suggested that the side of occurrence of accessory CL did not differ between primiparous and multiparous cows after induced ovulation with GnRH on d 5 post-ovulation (64.9 % vs. 65.9 %, $P = 0.91$). Our outcomes seem to agree with these findings as we did not observe an effect of treatment on side of accessory CL. Still, more primiparous cows had accessory CL ipsilateral to the original CL than multiparous (Table 2.1) across all cows with accessory CL. The physiology behind this effect of parity on side of accessory remains unclear.

The average ovulatory follicle diameter did not differ between treatments. Thus, the subsequent CL formed from these follicles should develop in a similar fashion (Vasconcelos et al., 2001). This allowed for interpretable comparisons between post-ovulation treatments with hCG effects on the original CL. Direct effects of hCG on luteal cell population have been confirmed to increase number and size of steroidogenic luteal cells, greater CL volume, and higher P4 in ewes treated with 300 IU of hCG intravenously (Farin et al., 1988; Schmitt et al.,

1996a). Although this study did not investigate luteal cell population, comparable outcomes were observed on both original and combined CL volumes. Cows in treatment D2&5 and D5 had greater volume of the original CL compared to controls. Similarly, cows treated with hCG on d 2 had increased serum P4 on d 5 post-ovulation (Figure 2.4) and increased original CL volume on d 10 post-ovulation (Figure 2.1). This agrees with other reports that have presented positive effects of hCG on volume of original CL when cows and heifers were treated early in the estrous cycle. Cabrera et al. (2021) recently demonstrated that treatment with ≥ 2,000 IU of hCG on d 7 of the estrous cycle increased existing CL volume in lactating dairy cows. In a comprehensive study, Maillo et al. (2014) had reported similar findings in which a single treatment with 3,000 IU of hCG on d 2 or 4 of the estrous cycle increased original CL tissue between d 6 and 13 of the cycle in beef heifers. Both studies have also reported increased circulating P4 in response to treatment despite the absence of accessory in heifers treated in d 2 in the later study. This indicates that hCG not only acts by increasing number of accessory CL but also by increasing the output of P4 via luteotropic effects on the developing original CL.

Analogous results are demonstrated on combined volume CL (Figure 2.2) which allows determination of effect of treatment on CL volume with additive effect of CL . Among all cows enrolled in the study, those treated with hCG had greater combined CL volume compared to controls (Figure 2.1). When analyzing the effect of treatment on cows with single ovulation and without accounting for those with ovulation in response to the D2 hCG, similar outcomes were observed. However, tendencies for difference between D2 and controls and D2&5 and D5 were identified (Figure 2.1). Clearly, the luteotropic effects of hCG on CL volume is additive of both formation of an accessory CL and its effects on the original CL. Moreover, some cows (8/9) also benefited from repeated treatment with hCG (d 2 and 5) which has allowed effects on the

original CL, ovulation of some cows soon after deviation of the first-wave dominant follicle, and a second ovulation in response to treatment on d 5.

Treatment with hCG post-ovulation increased serum P4 in parallel with luteotropic effects on CL volume. Our findings are in agreement with several other studies (Schmitt et al., 1996a; Santos et al., 2001; Rizos et al., 2012; Nascimento et al., 2013) that have reported greater serum P4 associated with increased CL volume following treatment with hCG post-ovulation. Treatment D2&5 and D5 induced greater average serum P4 on d 19 and 20 after AI/ovulation than treatment D2 or controls. Yet, our study partially differs from the data reported by Maillo et al. (2014). In this study, there was not enough evidence for difference in levels of serum P4 between treatment D2 and controls despite the effects of CL volume.

In summary, our data confirms that treatment with hCG on d 2 and 5 or on d 5 post-ovulation are adequate strategies to increase endogenous serum P4 during the pre-attachment phase of embryonic development by inducing the formation of accessory CL and increasing the volume of existing CL in lactating Holstein cows. However, treatment with hCG on d 2 post-ovulation did not increase serum P4 despite increasing the volume of existing CL or inducing the formation of accessory CL in a smaller percentage of cows. Other studies demonstrated a positive association between post-fertilization P4 levels and development of bovine concepti (Carter et al., 2008; Clemente et al., 2009; Forde et al., 2009). Yet, to the best of our knowledge, no relationship with conceptus attachment has been examined. Altogether, our findings support the use of hCG post-ovulation as a model to determine how time to conceptus attachment is affected after conceptus development under greater P4 or presence of accessory CL.

APPENDIX

Table 2.1. Effect of treatment with 3,000 IU of hCG on day 2 (D2), 2 and 5 (D2&5), 5 after ovulation (D5) or no treatment (Control) post-ovulation, and parity (primiparous, "P"; multiparous, "M"), on number and side of accessory CL (aCL) in lactating Holstein cows (n = 325) with single ovulations following the final GnRH of Double-Ovsynch and Ovsynch.

		Treatment [% (n)]					Parity [% (n)]		
Cows with aCL on d 10 post-ovulation	*Total* [% (n)]	**Control**	**D2**	**D2&5**	**D5**	*P* values	**P**	**M**	*P* values
≥ 1	51.1 (325)	0.0[a] (83)	24.7[b] (81)	89[c] (82)	92.4[c] (79)	< 0.001[2]	58.2[a] (91)	48.3[a] (234)	0.10[1]
Ipsilateral aCL	43.4 (166)	0 (0)	45[a] (20)	35.6[a] (73)	50.7[a] (73)	0.18[4]	54.7[a] (53)	38[b] (113)	<0.05[3]

[a-c] Different letters indicate difference in Chi-square test for homogeneity of proportions between treatments or parities ($P < 0.05$).
[1-2]*P* values for Chi-square test for homogeneity of proportions for presence of at least one accessory CL among treatments or parities.
[3-4]*P* values for Chi-square test for homogeneity of distribution of accessory CL by side relative to the single ovulation induced by GnRH among treatments or parities.

Figure 2.1. Effect of treatment with 3,000 IU of hCG on day 2 (D2), 2 and 5 (D2&5), 5 (d5) post-ovulation compared to no treatment (Control) on volume of the original CL on d 10 post-ovulation in all synchronized lactating dairy cows (n = 368) and in cows with only single ovulations* following Double Ovsynch or Ovsynch (n = 301). Data are presented as mean ± SEM. Different letters represent differences of least square means ($P < 0.05$).

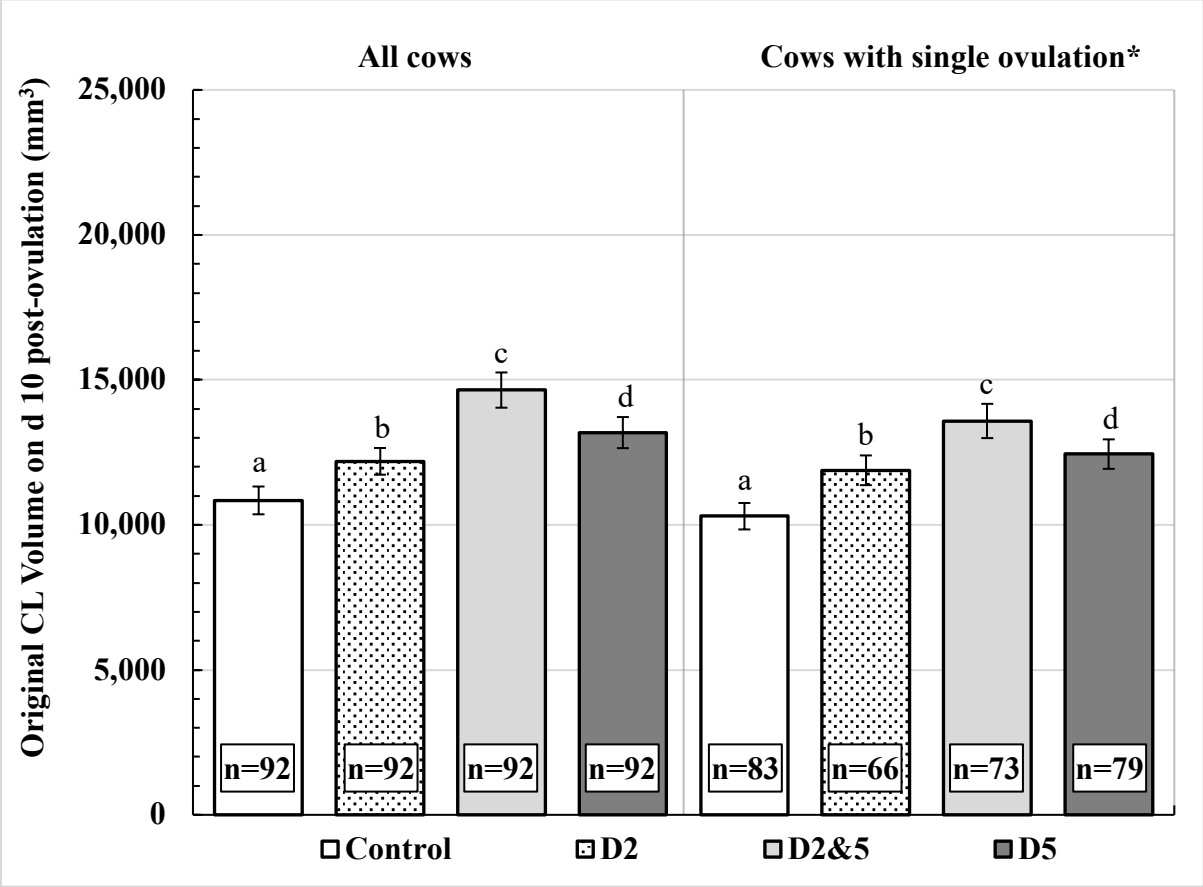


Figure 2.2. Effect of treatment with 3,000 IU of hCG on day 2 (D2), 2 and 5 (D2&5), 5 (d5) post-ovulation compared to no treatment (Control) on combined CL volume on d 10 post-ovulation in all synchronized lactating dairy cows (n = 368) and in cows with only single ovulations* following Double Ovsynch or Ovsynch (n = 301). Data is presented as mean ± SEM. Different letters represent differences of least square means ($P < 0.001$). Data are presented as mean ± SEM. Different letters represent differences of least square means ($P < 0.001$). Brackets indicate tendencies between least square means ($P \leq 0.10$).

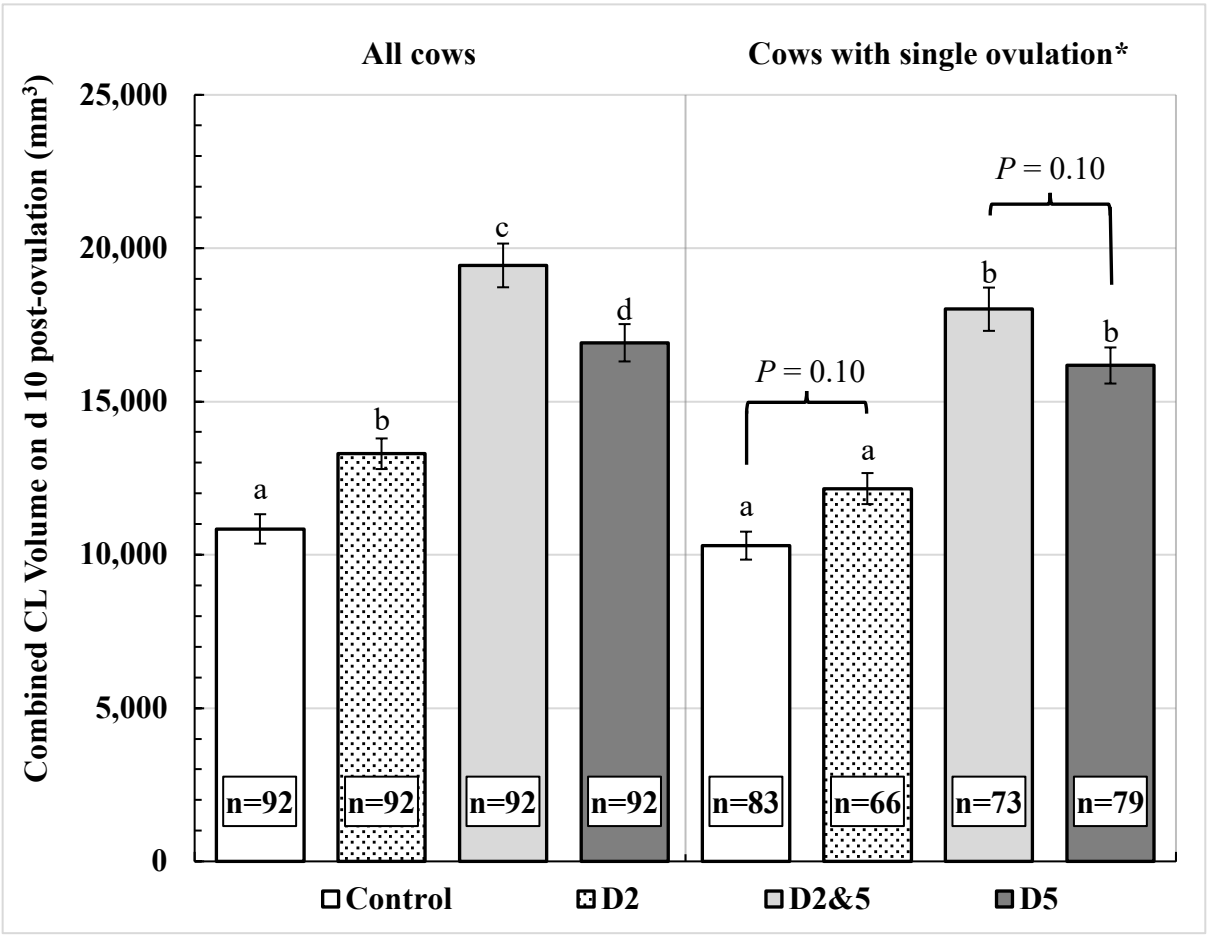


Figure 2.3. Effect of treatment with 3,000 IU of hCG on day 2 (D2), 2 and 5 (D2&5), 5 (d5) post-ovulation compared to no treatment (Control) on average serum concentrations of P4 on d 19 and 20 post-ovulation in all synchronized lactating dairy cows (n = 229) and in cows with only single ovulations* following Double Ovsynch or Ovsynch (n = 198). Data are presented as mean ± SEM. Different letters represent differences of least square means ($P < 0.01$).

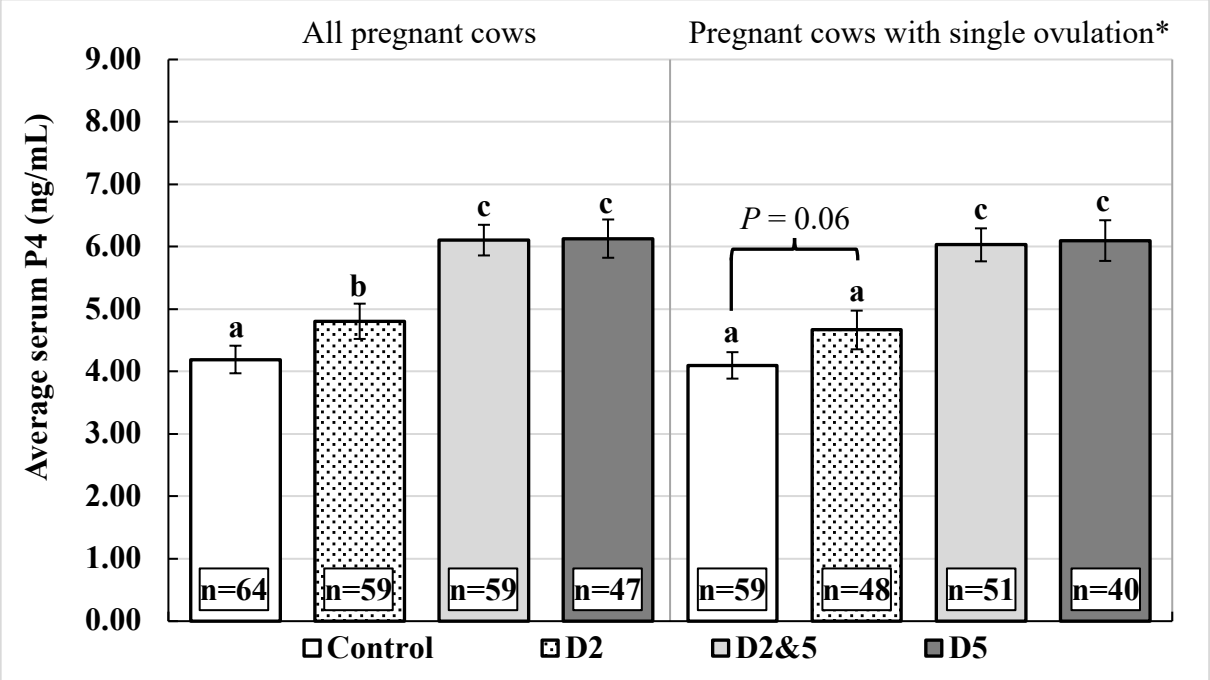


Figure 2.4. Effect of treatment with 3,000 IU of hCG on day 2 (D2) post-ovulation compared to no treatment (Control) on average serum concentrations of P4 on d 5 post-ovulation in synchronized lactating dairy cows (n = 251) with no accessory CL following treatment with hCG on day 2. Data are presented as mean ± SEM.

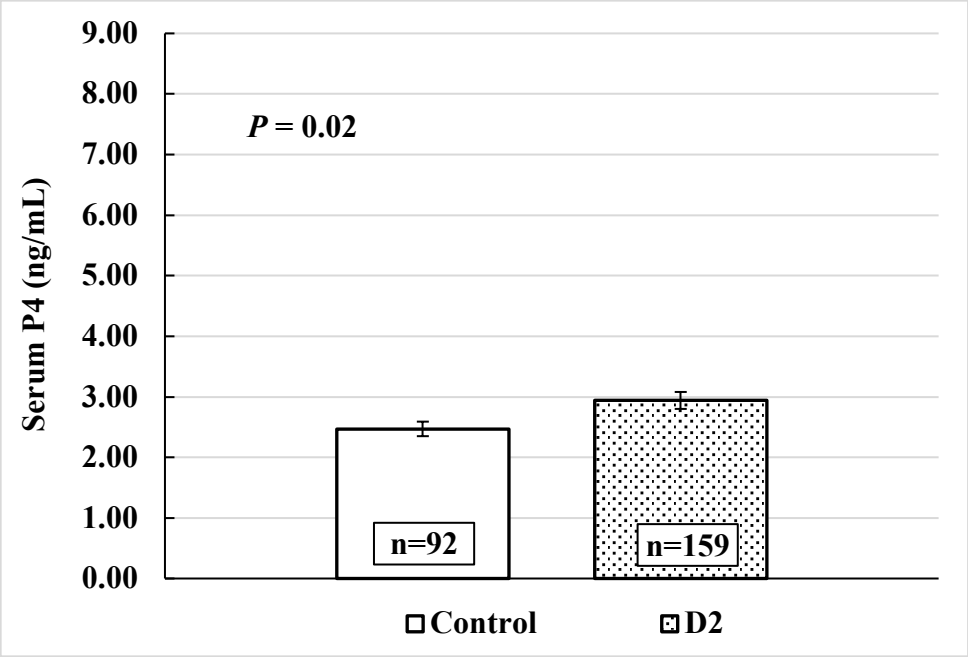
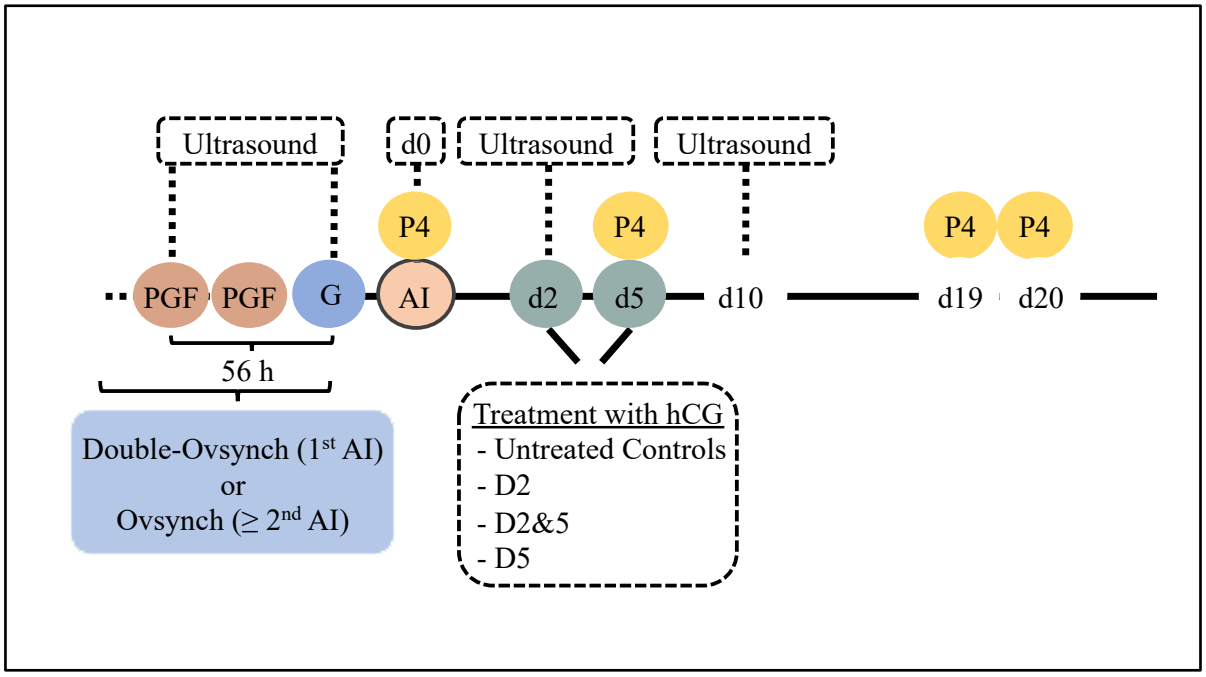


Figure 2.5. Experimental design. All cows were synchronized with either Double-Ovsynch or Ovsynch and examined via ultrasound on the final portion of the synchronization protocol (d of $PGF_{2\alpha}$ and GnRH). On d 0 (d of ovulation and AI), synchronized cows were blocked by parity and service and randomly assigned into one of four treatments: 3,000 IU of hCG on day 2 (D2), hCG on d 2 and 5 (D2&5), hCG on d 5 post-ovulation (d5), or no treatment (Control). All cows were examined via ultrasound on d 2 and 10 post-ovulation. Samples for serum P4 were collected on d 0, 5, 19, and 20 post-ovulation.



CHAPTER 3

EFFECT OF INDUCING ACCESSORY CL DURING EARLY EMBRYONIC DEVELOPMENT ON TIME TO CONCEPTUS ATTACHMENT IN LACTATING DAIRY COWS: PART II. IMPACT OF INCREASED P4 ON CONCEPTUS ATTACHMENT

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INTRODUCTION

Timely conceptus signaling and attachment are essential for establishment and maintenance of pregnancy in cattle (McCracken et al., 1984). During pre-attachment stages, the developing conceptus must signal its presence in the uterus through the secretion of interferon-tau (IFNT) to prevent luteolysis and sustain production of progesterone (P4) by the corpus luteum (CL). This will ensure uterine nourishment and further embryonic development towards term (Robinson et al., 1999). The histotroph, produced by endometrial glands, is required for pre-attachment embryonic growth and is directly affected by P4 (Guillomot et al., 1981). Post-ovulatory rising levels of P4 activate endometrial genes involved with synthesis and secretion of embryotropic substances that collectively compose the histotroph. This results in an indirect stimulatory effect of P4 on embryonic elongation(Forde et al., 2009). Studies associated greater risk of early pregnancy losses to lower post-ovulation levels of P4 in lactating dairy cows when compared to heifers (Sreenan et al., 2001; McNeill et al., 2006; Forde et al., 2009). In parallel, other studies reported a positive relationship between occurrence and side of accessory CL and progression of pregnancy (Baez et al., 2017; Monteiro et al., 2021).

A paucity of information exists to gain an understanding of how P4 impact on embryo elongation may influence the chances for, or time to, conceptus attachment. Measurement of PAGs, and more specifically pregnancy-specific protein B (PSPB), have been investigated as strategies to detect early pregnancy in dairy cattle (Middleton and Pursley, 2019; Filho et al., 2020). PSPB is synthesized by a lineage of trophoblastic cells, giant binucleate cells (BNCs), that migrate from the trophectoderm to the endometrial epithelium during conceptus attachment in ruminants (Guillomot et al., 1981). Studies have detected presence of PAGs in maternal serum as early as d 15 but consistently after d 20 of pregnancy (Sasser, 1986; Green et al., 2000). This

time of detection coincides with findings indicating that BNCs undergo morphological changes upon migration and fusion with the endometrial epithelium. It has been demonstrated that these cells lose a significant amount of cytoplasm via exocytosis of granules containing glycoproteins through the establishment of conceptus attachment (Wathes and Wooding, 1980; Wooding, 1992; Austin et al., 1999). Yet, no direct correlation between detection of PAGs and the determination of time of attachment nor of its relationship with P4 have been studied. This essential phase of embryonic development might contain key information that could benefit overall fertility of dairy cows.

Daily PSPB measurements prior to attachment and in non-pregnant cattle have significant variation between but not within cattle (Martins et al., 2018; Middleton and Pursley, 2019). The minimal variation day to day within individual cows allows for a robust model to determine time to increases in PSPB, and thus, conceptus attachment.

We hypothesized that increasing luteal mass and serum P4 during the pre-attachment phase of embryonic development would favor development and reduce the time to conceptus attachment in lactating dairy cows. In Chapter 2, we demonstrated that treatment with hCG on d 2 and 5 or on d 5 post-ovulation are adequate strategies to induce accessory CL and increase endogenous serum P4 during the pre-attachment phase of embryonic development in lactating Holstein cows. Therefore, this study aimed to investigate the effects of treatment with hCG on d2, d5, and d2 and 5 post-ovulation on time to conceptus attachment as a function of temporal changes in PSPB concentration lactating Holstein cows.

MATERIALS AND METHODS

Cows and Housing

This experiment was conducted at Nobis Dairy Farms in St. Johns, MI, between January and April of 2020. The herd consisted of approximately 1,100 Holstein cows milked 3-times a day with rolling herd average 13,971 kg of milk. Cows were housed in ventilated freestall barns with grooved concrete floors, head locks, and free access to food and water. A TMR was fed once daily and consisted of alfalfa and corn silage and concentrates meeting or exceeding nutrient requirements for lactating dairy cows (NRC, 2001).

All procedures in this experiment were performed by trained students and laboratory personnel and approved by the Institutional Animal Care & Use Committee at Michigan State University prior to execution.

Experimental Design

Power analysis indicated that 90 cows per treatment would be sufficient to detect a difference of 2 d to conceptus attachment with a standard deviation of $\pm$ 1 d to minimize a type II error $\leq$ 20 % at a significance level of $\leq$ 0.05. Weekly cohorts of lactating Holstein cows (n = 368) were synchronized with Double-Ovsynch (1st service; Souza et al., 2008) or resynchronized with Ovsynch (2nd service or greater; Brusveen et al., 2008) prior to enrollment in the study. The initial GnRH (86 μg gonadorelin acetate; Fertagyl®, Merck Animal Health, Kenilworth, NJ) of Double Ovsynch started at 48 $\pm$ 3 DIM. Timed-AI was performed in all cows 16 h after the last GnRH of Double Ovsynch (1st service; 77 $\pm$ 3 DIM) or Ovsynch in 2nd+ service cows that were resynchronized. All $PGF_{2\alpha}$ administrations utilized 500 μg cloprostenol sodium (Estrumate®, Merck Animal Health, Kenilworth, NJ).

As a reference, day of ovulation was considered the most critical point of measurement for calculation of time of fertilization due to the short lifespan of the oocyte (Szollosi, 1973; Hunter, 1985). Day of ovulation in this study was calculated as 24 to 32 h (Pursley et al., 1995) after final GnRH of Double Ovsynch or Ovsynch. All outcomes in this study are related to days post-ovulation.

Cows with functional CL and at least one large (≥ 10 mm) follicle on the day of $PGF_{2\alpha}$ 56 hours prior to final GnRH of Double Ovsynch or Ovsynch were considered synchronized. CL function was determined by the presence of blood flow through the entire tissue of each CL via color Doppler. Only synchronized cows were randomized in blocks by service and parity and assigned into one of four treatments: 3,000 IU of hCG (Chorulon®, Merck Animal Health, Kenilworth, NJ) on day 2 post-ovulation (D2), 3,000 IU of hCG on day 2 and 5 post-ovulation (D2&5), 3,000 IU of hCG on day 5 post-ovulation (D5), and no treatment (Controls; Figure 3.3).

Administrations of $PGF_{2\alpha}$, GnRH, and hCG were performed in either the semitendinosus or semimembranosus muscles using 3.5-cm, 20-gauge, single-use needles, and 3-mL, single-use syringes (BD, Franklin Lakes, NJ, USA).

Ovaries of all cows were evaluated and mapped on d 0, 5, and 10 post-ovulation via transrectal ultrasonography using a color Doppler MyLab™ GammaVET equipped with a 5-10 MHz multifrequency linear array probe (Esaote North America Inc., Indianapolis, IN). Synchronization of ovulation, and ovulation in response to treatments, were determined by the disappearance of the ovulatory follicle and presence of newly formed accessory CL on d 5 and 10 post-ovulation. Number and side of accessory CL were recorded. Pregnancy diagnosis on d 35, 63, or 100 post-ovulation was performed by the farm's veterinarian through transrectal ultrasonography.

Blood samples were collected from all cows for analysis of progesterone (P4) and pregnancy specific protein-B (PSPB) levels. Samples for P4 were obtained on days 0, 5, 19 and 20 post-ovulation. PSPB samples were collected daily from day 18 to 28 post-ovulation. Whole blood was collected via coccygeal vena puncture into vacuum tubes with clot activator and gel for serum separation (BD Diagnostics, Franklin Lakes, NJ). Samples were immediately placed in ice, transported to the laboratory, and incubated at 4° C for 24 h. All samples were centrifuged for 20 min at 2,016 x *g* at 4° C (Sorvall™ ST 40R, Thermo Fisher Scientific Inc., Waltham, MA). Aliquots of serum were transferred into 1.5 mL, labeled microtubes, and frozen at -20° C until assayed.

All frozen serum samples were shipped overnight to bioPRYN, bioTRACKING LLC, Moscow, ID where they were analyzed. P4 concentrations were determined by an enzyme immunoassay (EIA) developed bioTRACKING LLC as described in Chapter 2. Serum PSPB levels were measured using bioPRYN's commercially available PSPB ELISA assay kit as developed by Sasser (1986). Known PSPB samples were assayed in duplicate for quantification (8, 4, 2, 1, 0.5, 0.25, 0.125 and 0 ng/mL) of the standard curve. The lowest detected concentration among samples was 0.2 ng/mL. All samples from each cow were assayed in duplicates on the same plate along with a positive control and a negative control. Overall intra- and inter-assay CV were calculated within low and high control levels across 47 plates. Intra- and inter-assay CV were 2.25 and 4.02 %.

We identified a minimum threshold with the highest potential to determine conceptus attachment (CA) based on daily within-cow changes in serum PSPB (ng/mL; bioPRYN). Thresholds from 1 to 20 % were estimated and tested to predict the first day of continuous increase in serum PSPB. Conceptus attachment was determined in cows meeting two criteria: (1)

increase of equal to or greater than 12.5 % in serum PSPB from an individual baseline concentration followed by (2) two consecutive d of the same or greater daily increase in serum PSPB. An individual baseline concentration was determined as the average concentration between d 18 and 19 or as 0.2 ng/mL when the average was lower than the lowest detected concentration of 0.2 ng/mL with the bioPRYN assay. This model provided highest sensitivity and specificity for estimation of conceptus attachment relative to pregnancy status on d 35 post-AI/ovulation which consequently allowed estimation of time of conceptus attachment. That is, this model provided 100 % sensitivity, 75.1 % specificity, 100 % negative predictive value (NPV), and 79.9 % positive predictive value (PPV).

Data Management, Sample Size, and Statistical Analysis

Herd information such as weekly cohort, DIM, allocation group (pen), service, and parity were retrieved from the farm's data management software (PCDART, Dairy Records Management Systems, Raleigh, NC). All datapoints were entered weekly into one single Excel spreadsheet (Microsoft Corp., Redmond, WA).

Binomial outcomes were analyzed through logistic regression models using the LOGISTIC procedure in SAS (9.4, SAS Institute Inc., Cary, NC) under the LOGIT function. Final models considered the effect of treatments (D2, D2&5, D5, and Controls), service (1st service or resynchronization), parity (primiparous or multiparous), number of ovulations to the GnRH of the synchronization protocol (single or double), d 5 serum P4 quartiles (Q1-low to Q4-high), and average of d 19 and 20 serum P4 quartile (Q1-low to Q4-high), occurrence of accessory CL relative to the original CL (at least one or none), side of accessory CL relative to the original CL (ipsilateral or contralateral), d of conceptus attachment (from d 20 to 26), the occurrence of conceptus attachment (CA or no-CA), pregnancy status on d 35 post-AI/ovulation

(pregnant or non-pregnant), and the occurrence of pregnancy loss at any time after conceptus attachment (lost or maintained) as fixed effects. Two-way interactions were tested among all variables considering a hierarchical approach. Interaction terms and variables were only maintained in the final models if $P < 0.20$. Frequency and proportions were obtained using the FREQ procedure in SAS and tested by Chi-square test of homogeneity.

Continuous outcomes were analyzed through linear mixed models using the MIXED procedure in SAS. Linear models examined the same fixed effects and two-way interactions as those in the logistic models and also followed a hierarchical approach. Interaction terms and variables were only upheld in the final linear models if $P < 0.20$. All reported continuous means and SEM were obtained using PROC MEANS procedure in SAS. Repeated measurements of serum PSPB over d 18 through 28 post-ovulation were analyzed by a linear mixed model using MIXED procedure with a REPEATED statement in SAS. A final mixed model for repeated serum PSPB tested the occurrence of CA/pregnancy status on d 35 post-ovulation (CA & pregnant, conceptus attachment & and pregnancy loss, no-CA/non-pregnant) on serum PSPB, day of sample (d 18 through 28), and the interaction pregnancy status and day as fixed effects. A first-order auto-regressive (AR-1) covariance structure was used. Cows nested within pregnancy status groups were identified as SUBJECT within the repeated model.

All analyses applied 2-tailed tests. Effect of treatment on average d to conceptus attachment was compared to controls using pre-established orthogonal contrast (Control vs. D2; Control vs. D2&5; Control vs. D5). Significant differences were considered when P-values were ≤ 0.05 and tendencies were reported when P-values were ≤ 0.10. Tukey's adjustment was applied to all predetermined pairwise comparisons with > 2 covariates. Normality assumptions were determined by visual examination of datasets and though testing with Shapiro Wilk's test.

Data distribution information including quartiles were obtained using UNIVARIATE procedure in SAS (normal distribution as the null hypothesis).

RESULTS

Effect of treatment on time to conceptus attachment and pregnancy loss, and its association with parity

Treatment with hCG on d 5 increased average time to conceptus attachment ($P < 0.05$) of cows with conceptus attachment. On average ($\pm$ SEM), cows in D2 had conceptus attachment on d 20.85 $\pm$ 0.12, D2&5 on d 20.86 $\pm$ 0.13, D5 on d 21.17 $\pm$ 0.21 and controls on d 20.76 $\pm$ 0.09. Multiparous cows (21.07 $\pm$ 0.09 d) had longer ($P = 0.001$) time to conceptus attachment compared primiparous cows (20.48 $\pm$ 0.08 d). There was a treatment and parity interaction on time to conceptus attachment. Third and greater parity cows had increased ($P = 0.02$) d to conceptus attachment (21.9 $\pm$ 0.14 d) compared to first and second parity cows (20.6 $\pm$ 0.41 d) within D5 treatment.

Treatment affected the total percentage of cows with conceptus attachment. Treatment with hCG on D5 reduced the percentage of cows with conceptus attachment in comparison to controls. The percentage of conceptus attachment by treatment and total conceptus attachment was also associated with parity. Fewer primiparous cows had conceptus attachment after treatment on d 5 than controls. There was a tendency for decreased percentage of conceptus attachment in multiparous cows after treatment on d 5. Overall, a greater percentage of primiparous cows had conceptus attachment in comparison with multiparous (Table 3.1). No effect of treatment ($P = 0.50$) or parity ($P = 0.20$) was observed on pregnancy loss on d 35, 63, and 100 post-ovulation (Table 3.4).

Relationship of quartiles of serum P4 on d 5 and average on d 19 and 20, with average d to conceptus attachment in cows confirmed pregnant on d 35 post-AI/ovulation

Quartiles of serum P4 on d 19 and 20 were not associated with differences in average d to conceptus attachment across primiparous or multiparous cows with conceptus attachment. Yet, quartile of serum P4 on d 5 post-ovulation was negatively multiparous cows of the highest quartile of P4 on d 5 had reduced time to conceptus attachment in comparison to multiparous cows of the lowest quartile (Table 3.2).

Relationship of accessory CL and side with proportion of cows with conceptus attachment and pregnancy loss before d 100 post-AI/ovulation

Presence of at least one accessory CL was associated with lower percentage of multiparous cows with conceptus attachment (Table 3.3). Of all cows with single ovulation (n = 325), conceptus attachment was observed in 55.4 % (92/166) of those with accessory CL and in 66.7 % (106/159) of those with no accessory CL.

Side of accessory CL relative to the original CL did not affect the percentage of cows with conceptus attachment ($P = 0.32$). The occurrence of ipsilateral accessory CL was associated with lower percentage of pregnancy loss before d 100 post-AI/ovulation in primiparous cows (Table 3.3).

Relationship between time of conceptus attachment and pregnancy status on d 35 post-AI/ovulation

Of all cows enrolled in the study, 62 % had conceptus attachment (Table 3.1). Among cows confirmed pregnant at 35 d post-ovulation, 99 % (181/183) had conceptus attachment before or on d 22 and 1 % (2/183) had conceptus attachment on d 23 post-AI/ovulation. A large

proportion [24.9 % (46/185)] of the cows determined non-pregnant on d 35 post-AI/ovulation had conceptus attachment.

Cows with pregnancy loss between conceptus attachment and d 35 post-ovulation had greater ($P < 0.001$) time to conceptus attachment (d 21.7 $\pm$ 0.24) compared to cows that maintained pregnancy during that period (d 20.7 $\pm$ 0.05). Percentage of cows that had conceptus attachment on d 23 or later and were diagnosed non-pregnant (pregnancy loss) on d 35 post-AI/ovulation was greater than those with conceptus attachment on d 22, 21, or 20 (Figure 3.1). Cows with conceptus attachment $\geq$ 23 were more likely to experience pregnancy loss than cows with conceptus attachment on d 20 [odds ratio and 95 % C. I.: 23.84 (4.47, 127.22)] and on d 21 [21.0 (4.02, 109.50)]. Correspondingly, cows with conceptus attachment on d 22 were also more likely to experience pregnancy loss before d 35 than those with conceptus attachment on d 20 [4.67 (1.81, 12.10)] and on d 21 [4.11 (1.66, 10.18)].

Relationship between pregnancy status on d35 post-AI/ovulation and serum concentrations of PSPB

Pregnancy status on d 35 was associated with serum concentration of PSPB from a baseline to d 28 post-AI/ovulation. There was an interaction between d of sample and pregnancy status on d 35 (Figure 3.2). All cows had similar concentrations of PSPB at the baseline and on d 20 ($P = 0.90$). On d 21, cows with conceptus attachment and confirmed pregnant on d 35 had a tendency for greater PSPB compared to those that had pregnancy loss before d 35. On the same d, these had greater PSPB when compared to cows with no conceptus attachment on d 21. No difference in PSPB was observed on d 21 between cows with no conceptus attachment and cows that experience pregnancy loss ($P = 0.48$).

Beginning on d 22 through 28, a distinguishable pattern in serum PSPB was observed among pregnancy statuses. Pregnant cows had greater PSPB compared to both cows that had pregnancy loss after conceptus attachment and cows with no conceptus attachment. Similarly, cows that underwent pregnancy loss after conceptus attachment had lower PSPB compared to pregnant cows, but greater PSPB than cows with no conceptus attachment. Overall, serum PSPB remained at basal levels in cows with no conceptus attachment throughout the sampling period (Figure 3.2).

DISCUSSION

Main findings of this study support our understanding of the relationship of P4 and accessory CL with conceptus attachment in dairy cattle. Our findings indicated that (1) greater P4 on d 19 & 20 post-ovulation, occurrence of accessory CL, and greater parity decreased the percentage of cows with conceptus attachment, (2) greater serum P4 early, d 5 post-ovulation, was associated with reduced time to conceptus attachment in multiparous cows, (3) ipsilateral accessory CL relative to the original CL was beneficial to pregnancy in primiparous but not in multiparous cows, (4) time of conceptus attachment was key for progression of pregnancy, and (5) serum PSPB measured over time was indicative of pregnancy status on d 35 post-AI/ovulation.

Our hypothesis that treatment with hCG post-ovulation would increase luteal volume, serum P4 and reduce time to conceptus attachment in lactating Holstein cows was not proven. As reported in Chapter 2, treatments with hCG were effective in increasing luteal volume and serum P4 post-ovulation in lactating dairy cows, yet the D5 treatment actually reduced the percentage of cows with conceptus attachment.

Greater P4 on d 19 and 20 post-ovulation, occurrence of accessory CL, and parity decreased percentage of cows with conceptus attachment

Outcomes of this study indicated no relationship between luteal volume on d 10 post-ovulation and serum P4 on d 19 and 20 with time to conceptus attachment. It was also demonstrated that the occurrence of $\geq$ 1 accessory CL and greater P4 on d 19 and 20 due to treatment with hCG on d 5 post-ovulation were associated with reduced the percentage of cows with conceptus attachment. Yet, greater P4 during early conceptus development, d 5 post-ovulation, was associated with reduced time to conceptus attachment in multiparous cows. In the past, studies demonstrated that greater P4 post-fertilization was highly associated with higher probability of embryonic survival in dairy cows and heifers (McNeill et al., 2006; Parr et al., 2012). Others indicated that embryos that grew under greater serum P4 during the time of elongation were exposed to changes in the composition of the histotroph that led to larger elongated conceptuses and greater quantities of synthesized IFNT (Mann et al., 2006; Forde et al., 2009; Carter et al., 2010). Contrarily, two studies have reported potential detrimental effects of excessive P4 on the lifespan of CL in pregnant cows (Garrett et al., 1988b; O'Hara et al., 2014). These studies indicated that excessive P4 supplemented intramuscularly or via progesterone-releasing intravaginal devices (PRID) in early pregnancy stimulated premature pulsatile release of $PGF_{2\alpha}$ from the endometrium triggering luteolysis and shortening interestrus intervals. In sheep, Pope et al. (1995) demonstrated that the same daily dose of exogenous P4 that enhanced embryonic development also triggered luteolytic responses. Although our study did no assess time of luteolysis or interestrus intervals, we observed reduced conceptus attachment in one group of cows with greater serum P4 and CL volume. Perhaps, our findings may be related to similar physiological events leading to premature luteolysis in pregnant cows

when P4 exceeded optimal levels on d 19 and 20 post-ovulation. Yet, the findings on the relationship, or the lack of thereof, between late and early serum P4 and conceptus attachment indicated that greater early serum P4 favored conceptus development consequently reducing time to conceptus attachment.

In our study, a smaller percentage of multiparous cows had conceptus attachment in comparison to primiparous (Table 3.1). These findings are supported by several other studies that demonstrated negative association of greater parity and milk production with fertility (Pursley et al., 1997; Gröhn and Rajala-Schultz, 2000; Minela et al., 2021). Parity also confounded effects of treatment with hCG post-ovulation on fertility. Shabankareh et al. (2010) and Nascimento et al. (2013) reported increased conception rates across primiparous cows treated with hCG, but no differences in multiparous cows. Yet, Stevenson et al. (2007) demonstrated an increase in conception rate of second parity cows treated with hCG compared to controls. Past studies associated their findings of lower fertility in parous cows to greater milk production, increased dry matter intake, and steroid metabolism that led to lower oocyte and embryo quality (Sartori et al., 2002, 2004). A similar relationship between parity and fertility may have been demonstrated in our study considering our findings of lower conceptus attachment in multiparous cows despite greater P4 and accessory CL (Table 3.2; 3.3).

Side of accessory CL was associated with reduced pregnancy loss in primiparous cows

In our study, primiparous cows benefited from ipsilateral accessory CL as it reduced pregnancy losses before d 100 post-AI/ovulation (Table 3.3). Previous literature reported a positive relationship between occurrence and side of accessory CL and progression of pregnancy (Baez et al., 2017; Monteiro et al., 2021). In agreement with our findings, Monteiro et al. (2021) recently reported that regression of contralateral accessory CL occurred more frequently and

earlier in pregnancy than ipsilateral CL when investigating timing of accessory CL regression in early pregnancy. Their outcomes suggested that the same local mechanisms preventing regression of original CL protected ipsilateral CL luteolysis. Baez et al. (2017) also reported that a greater percentage of contralateral accessory CL underwent regression before d 67 of pregnancy in both primiparous and multiparous cows. Yet, such effect was not observed in multiparous cows enrolled in our study as pregnancy losses before d 100 post-AI/ovulation were not different in these cows. Differences in timing of accessory CL regression associated with limited sample size of cows with accessory CL may be the reason why no differences were observed in multiparous cows in our study.

Time to conceptus attachment was essential for successful pregnancy

Our study identified that greater pregnancy losses occurred in cows with longer time to conceptus attachment, as demonstrated in Figure 3.1. Previously, many studies demonstrated that conceptus attachment is a gradual continuous process in ruminants (Melton et al., 1951; Chang, 1952; Wathes and Wooding, 1980). Early conceptus-endometrium contact was reported as a facilitator for differentiation of trophectoderm cells into "giant binucleate cells" (Guillomot and Guay, 1982). In a past study, giant binucleate cells were identified after migration and fusion with endometrial epithelium cells that formed a transient syncytium between d 20 and 21 post-breeding (Guillomot et al., 1981). Previous work reported that pregnancy specific protein-B (PSPB or PAG-1) begins to rise in maternal circulation around this time (Sasser, 1986). We detected conceptus attachment as a function of changes in serum PSPB in 100 % of cows confirmed pregnant on d 35 post-AI/ovulation. However, 24.9 % (46/185) of cows diagnosed as non-pregnant on d 35 experienced pregnancy loss before d 35 according to our model. Across all cows with conceptus attachment $\geq$ d 23 or later (n = 12), 83 % (10/12) had

pregnancy loss before d 35 (Figure 3.1). Conversely, the vast majority of cows confirmed pregnant had conceptus attachment ≤ d 22 [99 % (181/183)]. This relationship of time of conceptus attachment and pregnancy loss could be justified on two bases: (1) delayed increase in PSPB (as an endocrine agent) led to failure in maintenance of the CL and occurrence of early luteolysis, and/or (2) PSPB (as marker of conceptus development and attachment) revealed abnormal conceptus attachment and placentation.

Studies have investigated the potential of PSPB as a hormonal agent in the process of conceptus attachment. Austin et al. (1999) tested the effect of different concentrations of PSPB obtained from d 120 cotyledons on the synthesis of uterine proteins by cultured endometrial explants. Their study revealed that PSPB induced synthesis of at least one alpha chemokine (GCP-2) also reported to be released in response to IFNT in early pregnancy (Staggs et al., 1998), suggesting a potential inhibitory role of PSPB on the release of $PGF_{2\alpha}$ during the establishment of pregnancy. Considering that expression of IFNT is abruptly ceased soon after conceptus attachment is initiated (Xavier et al., 1991), PSPB might have essential role in maintenance of the CL after conceptus-endometrium contact is established during the process of conceptus attachment. This would indicate that cows with longer time to conceptus attachment were more likely to experience early luteolysis and termination of pregnancy.

Pregnancy status on d35 post-AI/ovulation was preceded by different patterns of serum concentrations of PSPB

In a past study, expression of PAGs was demonstrated as highly variable between cattle in early pregnancy (Xie et al., 1991). Yet, several others have consistently demonstrated that PSPB rises in maternal circulation after conceptus attachment with very low day-to-day variability within cow (Green et al., 2000; Arnold et al., 2012; Pohler et al., 2016; Martins et al.,

2018; Middleton and Pursley, 2019). In agreement, our data revealed a consistent pattern of increase in serum PSPB beginning on d 20 post-AI/ovulation for cows that had conceptus attachment and maintained pregnancy until d 35.

In our study, a different pattern of increase in serum PSPB was also identified for cows with pregnancy loss before d 35 post-AI/ovulation (Figure 3.2). Cows that experienced pregnancy loss had different serum PSPB from cows with conceptus attachment that maintained pregnancy until d 35 beginning on d 22. These outcomes also agreed with others that demonstrated an association of levels of PSPB and progression of pregnancy, when investigating serum PSPB as a strategy to assess embryonic viability during the first trimester of pregnancy (d 30 to 41; (Semanbo et al., 1992). Pregnant cows were experimentally infected with *Actinomyces pyogenes* or treated with $PGF_{2\alpha}$. Their findings revealed that serum PSPB started to decrease gradually within the first 24 h after treatment in both groups. Results from this study reinforced that repeated measurement of PSPB within cow before and after conceptus attachment is an accurate method to assess time of conceptus attachment as well as pregnancy status.

In conclusion, treatment with hCG on d 5 post-ovulation reduced the percentage of cows with conceptus attachment and did not affect time to conceptus attachment despite increasing luteal volume and serum P4 post-ovulation in lactating dairy cows. Findings from our study suggested that early P4 (d 5 post-ovulation) but not late P4 was associated with time to conceptus attachment cows. Cows of first lactation benefited from reduced pregnancy losses before d 100 post-AI/ovulation associated with formation of ipsilateral accessory CL. These outcomes are supported by other studies that demonstrated protective effects of accessory CL on the lifespan of original CL and progression of pregnancy (Baez et al., 2017; Monteiro et al., 2021). However, multiparous cows with one or more accessory CL had lower conceptus attachment than those

with none, which is by supported other observations on the relationship between greater parity and reduced embryo quality (Sartori et al., 2002). Lastly, delayed time to conceptus attachment was highly associated with pregnancy losses before d 35 post-AI/ovulation. This revealed a positive association between maternal serum PSPB and progression of pregnancy which not only reiterated the efficacy of our model to identify conceptus attachment but also provided the basis for pregnancy diagnosis as early as d 22 of pregnancy with high accuracy.

APPENDIX

Table 3.1. Effect of treatment with 3,000 IU of hCG on day 2 (D2), 2 and 5 (D2&5), 5 (d5) post-ovulation compared to no treatment (Control) on % of lactating dairy cows (n = 368) with, and time to, conceptus attachment measured as increase of ≥ 12.5 % in serum PSPB from previous individual daily concentration followed by two consecutive d of the same or greater increase in serum pregnancy-specific protein B. *All cows with conceptus attachment were detected between d 20 and 26 post-ovulation.

	Day of conceptus attachment* (%)												
Treatment x Parity	**20**	**21**	**22**	**23**	**24**	**25**	**26**	*Total*	OR (95% CI)	*P*	*Overall*	OR (95% CI)	*P*
Controls											**69.5**		
P (n = 25)	40.0	36.0	4.0	0	0	0	0	**80.0**					
M (n = 67)	23.9	29.8	9.0	3.0	0	0	0	**65.7**					
D2											**64.1**		
P (n = 25)	52.0	20.0	0	0	0	0	0	**72.0**					
M (n = 67)	16.4	28.4	13.4	1.5	0	1.5	0	**61.2**					
D2&5											**64.1**		
P (n = 24)	50.0	25.0	4.2	0	0	0	0	**79.2**					
M (n = 68)	19.1	22.1	16.2	0	0	0	1.5	**58.9**					
D5											**51.1**	-0.6 (-1.02, -0.17)	0.01[3]
P (n = 23)	26.1	17.4	4.4	4.4	0	0	0	**52.3**	0.27 (0.08, 0.98)	0.04[1]			
M (n = 69)	17.4	20.3	4.4	2.9	1.4	2.9	1.4	**50.7**	0.54 (0.27, 1.1)	0.07[2]			
Total													
P (n = 271)	42.3	24.7	3.1	1	0	0	0	**71.1**					
M (n = 97)	19.2	25.1	10.7	1.8	0.4	1.1	0.7	**59.0**		0.03[4]			
Overall	25.3	25.0	8.7	1.6	0.3	0.8	0.5	**62.2**					

[1-3] *P* value for Chi-square test for homogeneity of proportions of total conceptus attachment compared to controls.
[4] *P* value for Chi-square test for homogeneity of proportions of total conceptus attachment between parities.

Table 3.2. Association of quartiles of serum P4 on d 5, and d 19 and 20 post-ovulation, on average d to conceptus attachment (d ± SEM) within primiparous (n = 69) and multiparous cows (n = 160).

	Average day to conceptus attachment (d ± SEM)							
	d 5 post-ovulation				**d 19-20 post-ovulation**			
Quartile of serum P4 (ng/mL)	**Primiparous**	*P*	**Multiparous**	*P*	**Primiparous**	*P*	**Multiparous**	*P*
Q1	**(1.03 - 1.88)**		**(0.34 – 1.67)**		**(0.47 – 3.43)**		**(0.60 – 3.49)**	
	20.5 ± 0.14	0.68	21.42 ± 0.19	0.02	20.59 ± 0.19	0.15	21.27 ± 0.24	0.19
Q2	**(1.88 – 2.93)**		**(1.67 – 2.25)**		**(3.79 – 5.69)**		**(3.57 – 5.15)**	
	20.41 ± 0.15		21.02 ± 0.18		20.39 ± 0.12		21.02 ± 0.16	
Q3	**(2.94 – 4.27)**		**(2.25 – 3.37)**		**(5.72 – 7.70)**		**(5.16 – 6.77)**	
	20.53 ± 0.12		21.17 ± 0.21		20.70 ± 0.19		21.22 ± 0.17	
Q4	**(4.27 – 8.40)**		**(3.40 – 8.40)**		**(7.70 – 8.40)**		**(6.85 – 8.40)**	
	20.47 ± 0.21		20.67 ± 0.11*		20.23 ± 0.11		20.77 ± 0.11	

P values for fixed effect of P4 quartile on d to conceptus attachment.

*Significant difference in comparison to the lowest quartile (Q1).

Table 3.3. Association of the occurrence and side of accessory CL (aCL) with percentage of lactating dairy cows [% (n)] with conceptus attachment (n = 325) and pregnancy loss between time of conceptus attachment and d 100 post-AI/ovulation in multiparous and primiparous cows.

	Conceptus attachment [% (n)]				Pregnancy loss before d 100 post-AI/ovulation [% (n)]			
Occurrence of aCL on d 10	**Primiparous**	OR (95% C.I.)	**Multiparous**	OR (95% C.I.)	**Primiparous**	OR (95% C.I.)	**Multiparous**	OR (95% C.I.)
None	71.0 (38)		65.3 (121)		14.8 (27)		31.6 (79)	
≥ 1	69.8 (53)	0.97 (0.61, 1.53)	48.7* (113)	0.71 (0.55, 0.92)	10.8 (37)	0.83 (0.4, 1.75)	29.1 (55)	0.94 (0.65, 1.37)
#Side of aCL on d 10								
Contralateral	70.8 (24)		45.7 (70)		23.5 (17)		34.4 (32)	
Ipsilateral	69.0 (29)	0.95 (0.53, 1.73)	53.5 (43)	1.17 (0.8, 1.71)	0.0* (20)	0.0017 (-, -)†	21.7 (23)	0.73 (0.4, 1.35)

* Significant difference in X^2 homogeneity test within parity ($P < 0.05$).

OR = Odds ratio and 95 % confidence interval for comparisons within parity, † calculation of confidence interval affected by quasi-complete separation of data points.

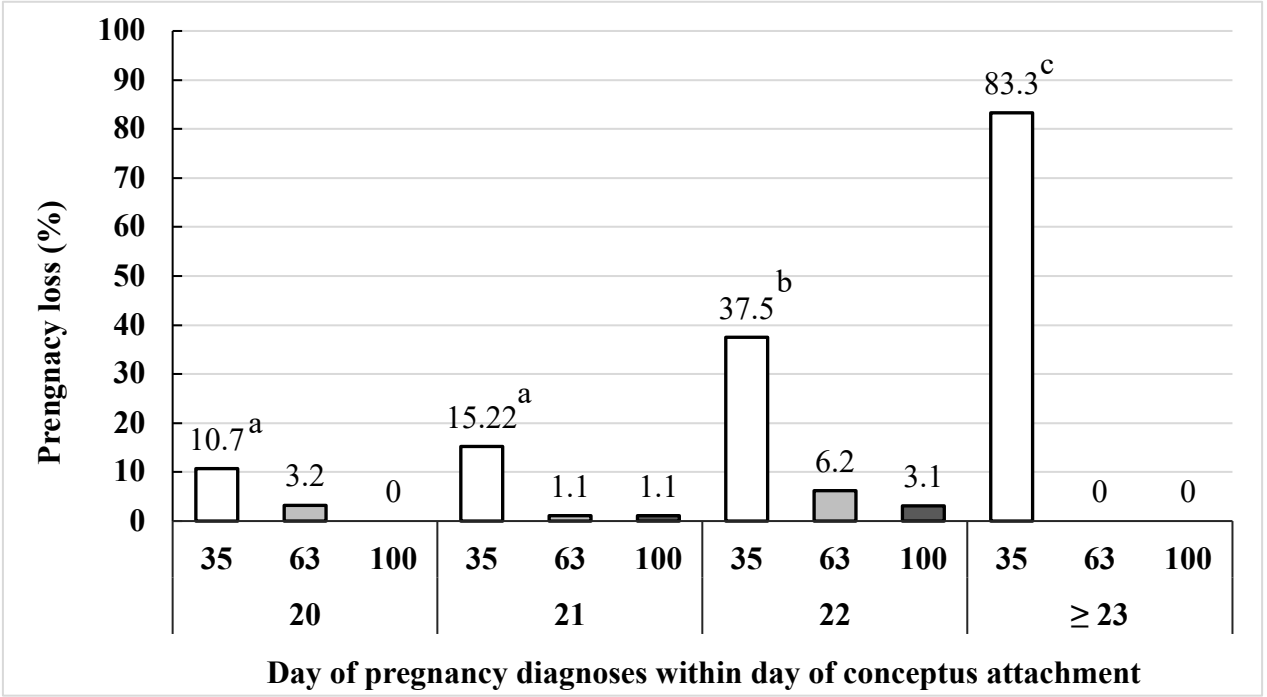
Relative to CL at beginning of cycle induced with final GnRH of Double Ovsynch or Ovsynch.

Table 3.4. Effect of treatment with 3,000 IU hCG on day 2 (D2), 2 and 5, 5, or no treatment (Control) on pregnancy loss on d 35, 63, and 100 post-ovulation in primiparous (n = 69) and multiparous (n = 160) lactating dairy cows that had conceptus attachment (n = 229).

	Pregnancy loss after conceptus attachment (%)					
	d 35		d 63		d 100	
Treatment	**Primiparous**	**Multiparous**	**Primiparous**	**Multiparous**	**Primiparous**	**Multiparous**
Control	10	20.4	0.0	5.7	0.0	6.2
D2	11.1	26.8	0.0	3.6	0.0	0.0
D2&5	5.3	17.5	5.6	6.1	0.0	0.0
D5	16.7	34.3	0.0	0.0	0.0	0.0
Total	10.1	24.4	1.6	4.2	0.0	1.8

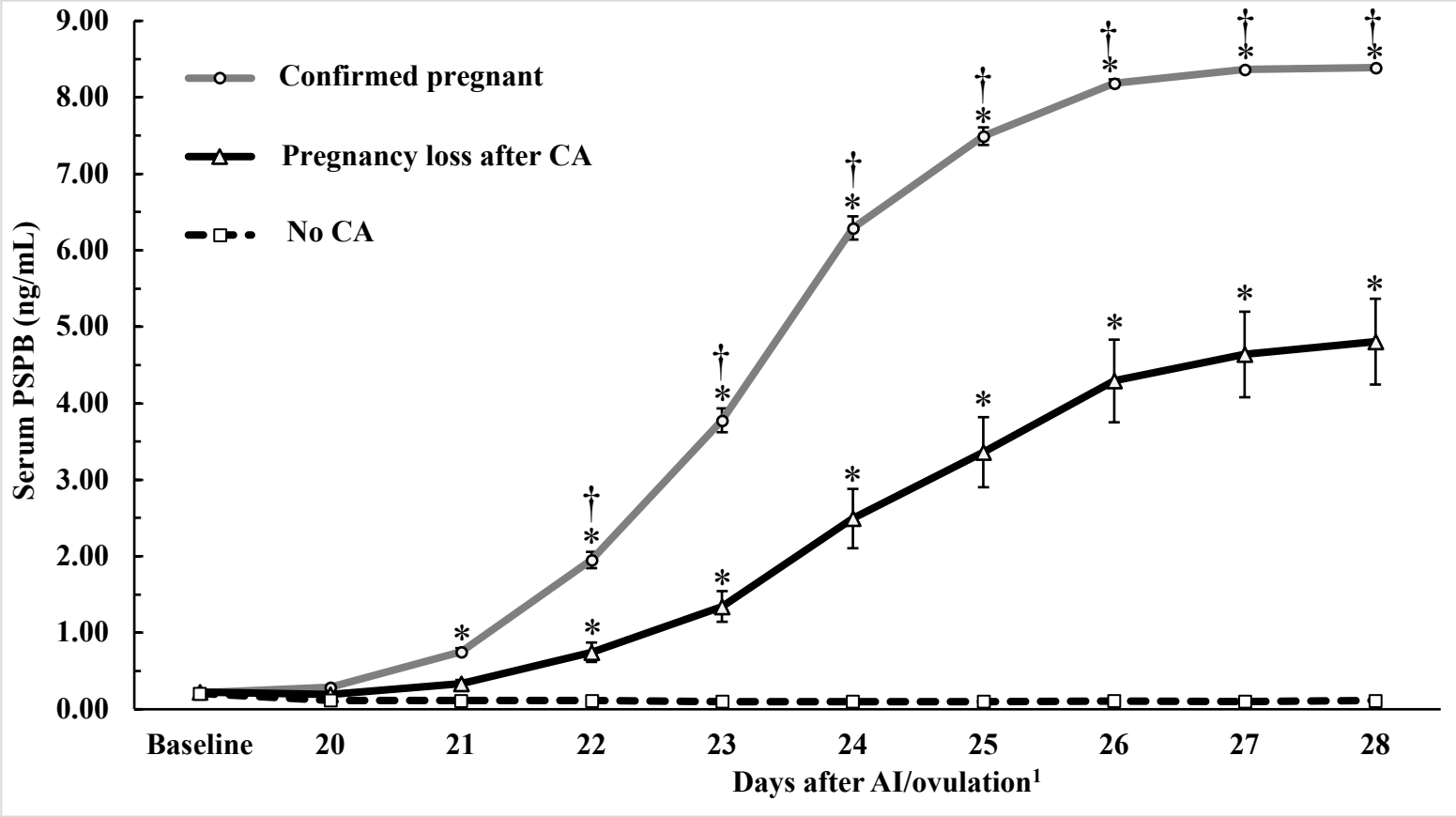
No difference observed in X^2 homogeneity test ($P > 0.05$)

Figure 3.1. Relationship between d of conceptus attachment and time of pregnancy loss in lactating cows with conceptus attachment (n = 229).



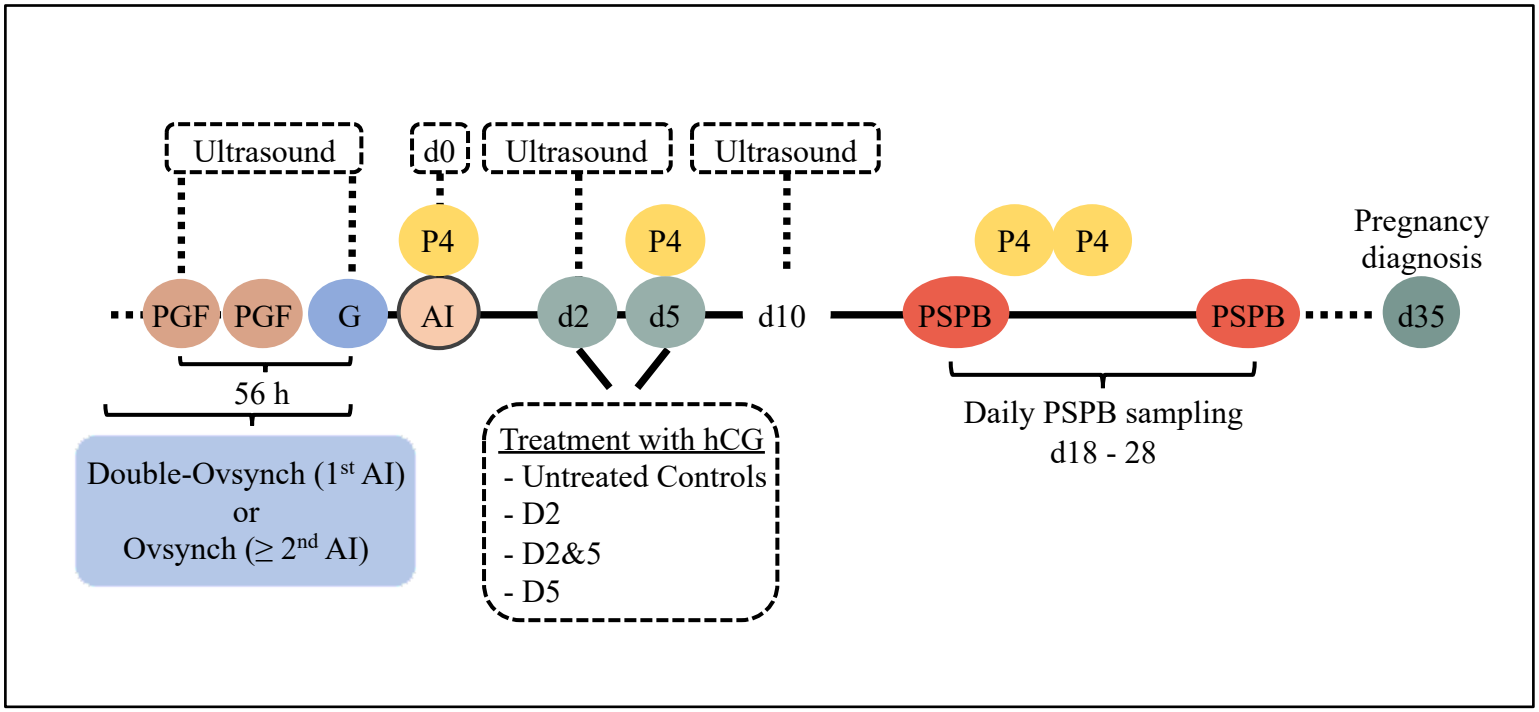
[a-c] Different letters represent difference in Chi-square test for homogeneity of proportions within d of attachment ($P < 0.05$).

Figure 3.2. Mean ± SEM serum concentrations (ng/mL) of pregnancy-specific protein B (PSPB) from baseline (average d 18 and 19 post-ovulation or lowest detectable concentration of assay[1] to d 28 post-ovulation in cows with conceptus attachment (CA) that were confirmed pregnant on d 35 post-ovulation (—○—), cows that had conceptus attachment and pregnancy loss before d 35 (—△—), and cows with no conceptus attachment (- -□- -).



[1]Lowest detectable concentration in the bioPRYN assay was (0.2 ng/mL).
[2]Pairwise comparisons of least square means among statuses within each day presented as: *difference in comparison with No-CA; †difference between confirmed pregnant (d 35) vs. pregnancy loss after conceptus attachment ($P < 0.05$).

Figure 3.3. Experimental design. All cows were synchronized with either Double-Ovsynch or Ovsynch and examined via ultrasound on the final portion of the synchronization protocol (d of $PGF_{2\alpha}$ and GnRH). On d 0 (d of ovulation and AI), synchronized cows were blocked by parity and service and randomly assigned into one of four treatments: 3,000 IU of hCG on day 2 (D2), hCG on d 2 and 5 (D2&5), hCG on d 5 post-ovulation (d5), or no treatment (Control). All cows were examined via ultrasound on d 2 and 10 post-ovulation. Blood samples for serum P4 were collected on d 0, 5, 19, and 20 and daily samples for PSPB were collected between d 18 to 28 post-ovulation. Pregnancy diagnoses were performed via ultrasound examination on d 35, 63, and 100 post-ovulation/AI.



CHAPTER 4

ECONOMIC IMPLICATIONS ASSOCIATED WITH POST-OVULATION hCG TREATMENT IN LACTATING DAIRY COWS

Significant work has been conducted to determine if post-ovulation hCG administration reduces pregnancy loss in lactating dairy cows. Outcomes from n = 7 studies (Table 1.1) that utilized hCG post-ovulation to induce accessory CL to increase progesterone during conceptus elongation indicated a 5 % increase in PR/AI, primarily due to increases in primiparous cows. In Chapters 2 and 3, treatment with hCG clearly demonstrated that treatment with hCG post-ovulation increased the number of accessory CL but did not increase the percentage of cows with conceptus attachment nor help to maintain pregnancies of cows that had conceptus attachment. However, there were clear differences in parity in this regard. Considering previous studies in Table 1.1 in addition to our outcomes, primiparous cows may benefit from hCG post-ovulation with regard to embryonic survival. Yet, the question whether these outcomes justify the level of investment associated with this strategy remains unanswered. Changes to reproductive programs demand diligent assessment of financial estimates just like other management decisions on dairy farms. Dairy producers must consider both costs and potential outcomes when comparing current vs. new strategies. As previously reviewed in this thesis, treatment with hCG post-AI/ovulation has produced inconsistent results in pregnancies per AI (P/AI) among the published literature. Hence, this chapter aims to present potential financial implications associated post-ovulation hCG treatment in lactating dairy cows estimated with an economic analysis software.

In Chapters 2 and 3, we reported a study that evaluated the effect of hCG treatment on time to conceptus attachment of lactating dairy cows. We observed that treatment with hCG on d 2 and 5 (D2&5) or on d 5 (D5) post-ovulation induced the occurrence of at least one aCL in a greater ($P < 0.01$) percentage of cows compared to those that received treatment on d 2 (D2) or controls (no treatment). The percentage of cows with conceptus attachment was lower ($P = 0.01$) in cows treated with hCG on d 5 compared to controls. Particularly within treatment D5, cows of

3rd and greater parity had greater time to conceptus attachment ($P = 0.03$). Later analysis revealed that cows with greater time to conceptus attachment had greater chances of experiencing pregnancy loss at any time between d of conceptus attachment and d 100 post-ovulation ($P < 0.05$). We also observed that the occurrence of at least one aCL decreased the percentage of cows with conceptus attachment among multiparous cows synchronized with Double-Ovsynch ($P < 0.05$). In contrast, the occurrence of at least one aCL ipsilateral to the original CL appeared to enhance embryonic survival compared to contralateral and control groups ($P < 0.05$). These outcomes, presented in greater detail in Table 3.3 of Chapter 3, were used for comparison of different scenarios considering the occurrence and side of occurrence of aCL within parities (primiparous and multiparous).

With regards to costs of the alternative strategy, the current price of hCG as well as costs with additional labor were considered. In August of 2021, the cost of a vial of Chorulon® (Merck Animal Health, Kenilworth, NJ) with 10,000 IU of hCG was approximately $50.00 in the United States. Therefore, a working dose of 3,000 IU would represent an expense of approximately $15.00 per cow per service in the alternative scenario. Furthermore, we estimated $1.00 per cow per service as the additional cost of labor for administration of hCG injections. All scenarios were compared within a single 1,100-head herd using the same parameters presented in Chapter 2. The average body weight was considered 1,500 lb. with involuntary culling of 28 %, 3 % incidence of stillbirth, and overall mortality rate of 4 %. A default lactation curve of 25,000 lb. of milk per cow per year as applied for comparisons among primiparous cows and a lactation curve of 29,000 lb. of milk per cow per year was used for multiparous cows. Female calf value was set as $250.00 and male calf value was set as $100.00. Milk price per cwt applied in the calculation

was $17.16. Finally, salvage value was fixed as $0.526 per lb. and heifer replacement value was set at $1,600.00.

Estimation of profit/loss using Dairy Repro$

Treatment on d 5 post-ovulation with hCG reduced herd profit when compared to controls that did not receive a post-ovulation treatment. The estimated loss in annual herd profit associated with treatment was -$112,750 for multiparous and - $97,570 for primiparous cows.

The occurrence of at least one aCL due to post-ovulation hCG treatment in either primiparous or multiparous cows reduced annual herd profits (Table 4.1) when compared to a standard reproductive program using Double-Ovsynch and Ovsynch. The loss in net value was estimated as $4,180 in primiparous cows and $83,490 in multiparous cows. Occurrence of an aCL contralateral to the original ovulation reduced fertility outcomes in both primiparous and multiparous cows compared to controls, thus is not economically viable. However, the creation of aCL ipsilateral to the original CL was projected to benefit annual economics of primiparous ($39,600) and but not multiparous cows (-$22,550) in specific scenarios when compared to controls. Unfortunately, we know of no way to only obtain ipsilateral aCL in primiparous cows following treatment post-ovulation with hCG.

Most studies in the literature would indicate that hCG can enhance embryonic survival in primiparous cows but not multiparous cows. Yet, our data suggests otherwise. We do not recommend using hCG in a way to induce post-ovulation aCL to improve reproduction in lactating dairy cows. These outcomes will be important to decision making on dairy farms. There are dairy farms utilizing hCG to induce post-ovulation aCL with the expectation of enhancing numbers of pregnancies. It is not clear how many farms are using this technology. Nevertheless,

we intend to publish the outcomes of this economic analysis so that farmers and their veterinarians can have access to this information to make informed decisions.

APPENDIX

Table 4.1. Predicted annual net value (USD) by parity (primiparous "P" or multiparous "M") gained by inducing ≥ 1 accessory CL (aCL), inducing ≥ 1 aCL ipsilateral to the original CL, or inducing ≥ 1 aCL contralateral to the original CL compared a current program to an alternative program in a 1,100-cow herd in the State of Michigan, USA, calculated via an economic analysis software (UWCU-DairyRepro$).

	Annual herd profit projected for alternative program (USD)					
	≥ 1 aCL		**Ipsilateral aCL**		**Contralateral aCL**	
Current Program	**P**	**M**	**P**	**M**	**P**	**M**
No aCL	-$4,180	-$83,490	$39,600	-$22,550	-$41,360	-$125,950
Contralateral aCL	.	.	$81,070	$103,510	.	.

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