ADVANCING THE PHARMACOLOGICAL CONTROL OF OVARIAN DEVELOPMENT TO ENHANCE FERTILITY OF LACTATING DAIRY COWS

By

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ABSTRACT

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Reproductive performance of lactating dairy cows is a key profit generator for dairy producers. During the last 6 decades, as milk production has increased significantly, pregnancy per artificial insemination (P/AI) of lactating dairy cows has decreased. Moreover, high milk production is associated with the increased metabolic clearance rate of progesterone (P4) and estradiol due to greater feed intake and greater blood flow through the liver. Previous data from our laboratory and others have indicated that cows with the greatest circulating concentrations of P4 during ovulatory follicle development had the greatest P/AI. Furthermore, timed-AI programs that control follicle and corpus luteum (CL) development increase circulating concentrations of P4 during the growth of the ovulatory follicle and synchronize ovulation, and improve fertility of lactating dairy cows following timed-AI. The mechanism involved on the effects of levels of P4 during growth of the ovulatory follicle on fertility remains unclear.

Our overall hypothesis of this dissertation is that treatments that increase serum concentrations of P4 during the growth of the ovulatory follicle increase P/AI and reduce pregnancy losses in lactating dairy cows. The three main objectives are: (1) determine the effect of three fertility treatments on serum concentrations of P4 during the growth of the ovulatory follicle and P/AI of lactating dairy cows; (2) develop a simpler fertility treatment for dairy operations limited by labor or other logistical constraints that cannot use currently fertility treatments; and (3) determine the effect of high vs. low P4 during ovulatory follicle development on fertility of lactating dairy cows.

Research in Chapter 3 focused on the comparisons of fertility treatments that increase the percentage of cows with an accessory CL during the growth of the ovulatory follicle. We hypothesized that fertility treatments that use pre-synchronization with $PGF_{2\alpha}$ and GnRH would increase: percentage of cows with an accessory CL at time of ovulatory follicle development, circulating levels of P4 and P/AI compared to pre-synchronization with only $PGF_{2\alpha}$. Results indicated that fertility treatments with pre-synchronization with GnRH were able to increase percentage of cows with accessory CL; However, P/AI was similar to a pre-synchronization with only $PGF_{2\alpha}$. Interestingly, enhancing P4 during growth of the ovulatory follicle was associated with enhanced PAI of in a large number of lactating dairy cows (~2500). In Chapters 4 and 5 experiments, we hypothesized that combining $PGF_{2\alpha}$ and GnRH (PG+G) in a pre-synchronization strategy one week before Ovsynch would result in similar P/AI compared to other fertility treatments. Results indicated that our simpler pre-synchronization program had similar outcomes compared to fertility programs. Thus, it may offer a reasonable alternative to more complex fertility programs to enhance P/AI. The hypothesis of Chapter 7 study was that reduced circulating levels of P4 during ovulatory follicle would decrease P/AI and increase pregnancy loss during gestation. P4 was manipulated to reach high or low circulating concentrations during the pre-dominance phase and dominance phase of the ovulatory follicle. Low P4 during ovulatory follicle development increased double ovulation rate in lactating dairy cows. This resulted in a higher P/AI at d 23 post-AI. However, low P4 also created greater losses during post-attachment period to 56 days post-AI. Most of these losses post-attachment was primarily due to unilateral double ovulation/ twins.

I dedicate this dissertation to my family, especially two people: my grandmother, Maria Júlia da Costa Salgueiro Nascimento, for a life of love and devotion to her family, and to my wife, Ayra C. K. de Almeida, for her support, friendship, comprehension and love during this entire journey.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

AI	artificial insemination
CL	corpus luteum/corpora lutea
CR	conception rate
CV	coefficient(s) of variation
d	day (s)
DPC	days post coitum
DF	dominant follicle(s)
DIM	days in milk
E2	estradiol
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
h	hour (s)
im	intramuscular
LH	luteinizing hormone
ng	nanogram
P4	progesterone
P/AI	pregnancy per artificial insemination
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
RIA	radioimmunoassay
TMR	total mixed ration

CHAPTER 1

INTRODUCTION

THE PROBLEM OF INFERTILITY IN HIGH PRODUCING LACTATING DAIRY COWS

Reproductive efficiency is a crucial factor affecting profitability and sustainability of dairy farms. During the last decades, fertility of lactating dairy cows has decreased at an alarming rate. One of the best indicators of fertility of cattle is conception rate or pregnancy per artificial insemination (P/AI). P/AI is the number of cows pregnant by the number of cows inseminated. P/AI of lactating dairy cows inseminated following estrus detection were approximately 65% in the 1950's [1], 50% in 1985 [2], 42% in 1999 [2] and currently are about 35% [3,4]. However, fertility of nulliparous heifers (females that have never calved) remained high and currently P/AI of dairy heifers are approximately 60% [5]. One of the major obstacles for increasing P/AI in high producing dairy cows is the high incidence of embryonic mortality and pregnancy losses during the gestation period. Approximately 50% of embryos are lost between fertilization and 42 d of gestation (early embryonic death) [6]. The incidence of embryonic loss in producing lactating dairy cows is greater compared with nulliparous heifers.

ASSOCIATION BETWEEN LOW CIRCULATING LEVELS OF STEROID HORMONES AND INFERTILITY IN LACTATING DAIRY COWS

While the exact causes of low P/AI and high pregnancy losses are unknown, the decline in fertility in lactating dairy cows has been attributed to increased milk production and dry matter intake [8]. In order to produce high yields of milk, lactating cows require greater dry matter intake compared to heifers or beef cows that result in increased blood flow passing through the liver and thus greater steroid metabolism [8-11]. Therefore, lactating dairy cows have lower circulating steroid concentrations than heifers, despite having larger ovulatory follicles and corpora lutea (CL)[12,13]. Lower circulating concentrations of estradiol (E2) and progesterone

(P4) have several potential physiological consequences that may impair fertility of lactating dairy cows [14]. Low circulating concentrations of E2 was associated with a decrease in estrus detection rates, causing a reduction of reproductive efficiency. A study by Washburn et al. [2] reported a decrease from 50.9% in 1985 to 41.5% in 1999 for estrus detection rates in Holstein dairy herds in southeastern USA. This reduction of estrus detection rate was associated with the increase in milk production per cow during this period and, consequently, with the increase in the metabolism of E2. Lopez et al. [15] showed the negative effect of high milk production on duration of estrus. In their study, high producing cows had lower circulating concentrations of E2 and shorter duration of estrus compared to cows with lower milk production. The probability of detecting a cow in estrus is less for cows with shorter duration of estrus. When the estrus of a cow is missed, and the cow is not inseminated, an entire estrous cycle (~ 23 d of length) will have to pass until this cow have another chance to be detected in estrus and inseminated. This increases the number of days that cows are not pregnant and represents economic losses for the dairy producer.

The decrease in circulating concentrations of P4 has also been related with the reduction in P/AI following artificial insemination in lactating dairy cows. In 1973, Folman et al. [16] was the first to identify a positive relationship between serum concentrations of P4 during the estrous cycle and fertility of lactating dairy cows following estrus detection / AI. In their study, they found that cows that conceived after first insemination had significantly greater levels of circulating P4 4 to 15 days before estrus compared to cows that did not conceive. The results indicated a positive correlation between circulating P4 and P/AI after first insemination. However, since the number of cows in this study was small (n = 14), this result could be an effect of a Type II error. In 1983, Fonseca et al. [17] identified the importance of circulating P4

prior to AI on fertility of lactating dairy cows in a study with greater number of cows (n = 212). For each 1 ng/mL increase in circulating concentrations of P4 during the 12 d interval prior to AI, P/AI increased by 12.4 ± 3.7%. These were the first studies that suggested a role of progesterone prior to AI in P/AI in lactating dairy cows. This data used cows that were inseminated following estrus detection, without pharmacological manipulation of ovarian function. Most recently, studies using synchronization of ovulation programs also identified an effect of serum concentrations of P4 during the growth of the ovulatory follicle on P/AI.

EFFECT OF CIRCULATING CONCENTRATION OF PROGESTERONE DURING SYNCHRONIZATION OF OVULATION PROGRAMS ON FERTILITY OF LACATATING DAIRY COWS

Cows that are treated with synchronization of ovulation program but have low circulating concentrations of P4 during the growth of the ovulatory follicle, still have low P/AI [20-25]. Synchronization of ovulation programs are also known as timed-AI programs. Preliminary data from our laboratory shows a clear relationship between circulating concentrations of P4 during the growth of the ovulatory follicle during timed-AI programs, and fertility of dairy cattle. The part of the timed-AI program that controls the ovulatory follicle development is the Ovsynch protocol. Although timed-AI programs control follicle and CL development a large variation of serum concentration of P4 is found among cows during the Ovsynch protocol. Cows under Ovsynch with low circulating concentrations of P4 during the growth of the ovulatory follicle, have low P/AI [20-25]

Recent studies investigated the effect of increasing serum concentrations of P4 during Ovsynch protocol in lactating dairy cows [22,25,26]. Denicol et al. [25] tested the effect of a supplementation of P4 during the first follicular wave on fertility of dairy cows compared to

cows without P4 supplementation. In this experiment, cows were assigned to three different treatments. One treatment increased percent of cows with second follicular wave as the ovulatory wave, with two corpora lutea (CL). The other two treatments increased percent of cows with first wave as the ovulatory wave, with and without P4 supplementation (two intra-vaginal progesterone releasing device). Cows with P4 supplementation had increased circulating concentrations of P4 compared to cows without P4 supplementation in the first follicular wave. They also had similar concentrations of P4 compared to cows in the second wave. Cows in the first wave with P4 supplementation had increased P/AI 38 d after AI compared to cows without P4 supplementation. However, there were no differences in P/AI 66 d after AI. An important factor that was not taken into consideration for the analysis of these data was the ovulation to the first GnRH of the Ovsynch. Cows that do not ovulate for the first GnRH of Ovsynch, do not start a follicular wave at the same time compared to cows that do ovulate, having differences in ovulatory follicle ages. Therefore, control of the ovulatory follicle age and time that circulating levels of P4 was enhanced in relation to follicle development may had affected the results of this study.

Bisinotto et al. performed several studies to test if supplementation of P4 during Ovsynch would enhance circulating P4 of cows with or without CL (majority of cows without CL were considered anovular cows) [23,26,27]. In the first two studies, cows without CL at time of first GnRH of Ovsynch were randomly assigned to a modified Ovsynch (7 d [26] or 5 d [23] pre-luteolysis period) with or without P4 supplementation (2 P4 intravaginal devices - CIDR). Cows without CL that received P4 supplementation had greater circulating concentrations of P4 compared to cows with no P4 supplementation [23,26]. This increase in circulating concentrations of P4 resulted in greater P/AI 34 and 62 d after AI [23,26]. Hence, enhancing

circulating concentrations of P4 during the first follicular wave increased fertility of anovular cows. In another study from the same laboratory, cycling cows with a CL were supplemented with P4 using only one CIDR during a regular Ovsynch protocol [27]. In this case, cows with P4 supplementation did not increase circulating concentrations of P4 during the period of follicle development compared to cows without P4 supplementation. Hence, there were no differences in P/AI between cows with or without P4 supplementation [27].

Although compelling evidence indicates a major role of serum concentrations of P4 during the growth of the ovulatory follicle on fertility of lactating dairy cows, there is still a lack of a study that demonstrate the cause/effect of circulating levels of progesterone on fertility of lactating dairy cows. The overall objective of this dissertation is to use pharmacological control of ovarian development to increase circulating concentrations of P4 during ovulatory follicle growth and optimize fertility of lactating dairy cows. The overall hypothesis of the present dissertation is that pharmacological control of ovarian development that increase serum concentrations of P4 during growth of the ovulatory follicle will enhance fertility of lactating dairy cows by reducing pregnant losses.

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SECTION 1

CONTROL OF FOLLICLE AND CORPUS LUTEUM DEVELOPMENT USING FERTILITY TREATMENTS IN LACTATING DAIRY COWS

CHAPTER 2

FERTILITY PROGRAMS FOR LACTATING DAIRY COWS, THEIR PHYSIOLOGICAL BASIS, AND THE FACTORS THAT ARE CRITICAL FOR THEIR SUCCESS

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ABSTRACT

Lactating dairy cows have unique reproductive parameters compared to when they were dairy heifers that result in distinctly different reproductive measurements and outcomes that can be partially overcome with pharmacological strategies. These parameters include circulating progesterone and estradiol concentrations, ovulatory follicle and corpus luteum diameter, incidence of anovulation and double ovulations, time in estrus, pregnancies per artificial insemination and pregnancy losses. Metabolic differences such as milk production and the effect of dry matter intake on blood flow to the liver appear to be key drivers of these differences in reproductive function. Circulating concentrations of progesterone are approximately half in lactating dairy cows compared to heifers. This marked difference in progesterone is likely the explanation for an increased size in diameter of the ovulatory follicle and incidence of double ovulations in cows vs. heifers. Differences in diameter of the ovulatory follicle may explain why cows have greater corpora lutea diameters compared to heifers. The increase in double ovulations appears to be a key driver in the increase in twinning and pregnancy loss as dairy heifers transition to primi- and multiparous cows. Reduced estradiol concentrations in cows at time of estrus helps to explain the decreased duration of estrus in cows vs. heifers. Concentrations of progesterone during growth of the ovulatory follicle may be a key driver in differences in pregnancies per AI in cows vs. heifers. The difference in circulating progesterone may be related to LH overstimulation of the oocyte/cumulus complex in cows compared to when they were heifers. Pharmacological strategies have been developed in lactating dairy cows to manipulate ovarian development to create a hormonal environment similar to that of heifers. Three primary strategies appear to increase fertility compared to artificial insemination following detected estrus. This review discusses how these three strategies manipulate ovarian

development similar to that of heifers and why compelling data indicate these programs should be referred to as “fertility programs.”

INTRODUCTION

The evolutionary role of the ovary is to produce oocytes capable of fertilization. The supporting cast of ovarian structures, follicles and corpora lutea, are responsible for the environment and production of oocytes and maintenance of pregnancy. The physiological outcomes of these structures in dairy cows change when lactation begins and is associated with negative reproductive outcomes [1]. Pharmacologic manipulation of ovarian structures can reverse these effects of lactation and enhance fertility [2]. The physiological basis for development of pharmacological programs to improve fertility of lactating dairy cows comes from studies that characterized key differences in reproductive indices in primiparous and multiparous cows (referred heretofore as “cows”) vs. nulliparous heifers (referred heretofore as “heifers”) [3-5]. Differences in circulating concentrations of progesterone appears to be the key driver of many of these indices. Lactating cows have reduced circulating concentrations of progesterone during the estrous cycle compared to heifers [3-5]. This results in greater number of pulses of LH during the luteal phase of the cycle and in turn drives the growth of larger dominant and ovulatory follicle diameters in cows vs. heifers [3,5]. These differences in progesterone and LH pulsatility also create differences in length of follicular waves [5]. In this case, cows with lower concentrations of progesterone have longer inter-wave intervals compared to heifers [5] due in part to increased numbers of LH pulses that drive the growth of a dominant follicle for longer periods. This, in turn, leads to more cows with an ovulatory follicle with a greater antral age (measured from onset of wave to ovulation) that developed under greater numbers of LH pulses compared to heifers. Heifers have greater chances to have three waves of follicle growth

during a slightly shorter estrous cycle if the second wave dominant follicle becomes atretic prior to endogenous luteolysis [6]. An increase in double ovulations as dairy heifers transition to primi- and multiparous cow appears to be a key driver in the increase in twinning [1,3,7,8]. It appears the increase in twinning has a significant impact on pregnancy loss [9,10]. Metabolic differences such as milk production and increased dry matter intake may explain these differences in reproductive function [1].

Reproductive inefficiency is an obstacle to dairy farm profitability and sustainability. During the past 50 years, reproductive efficiency of lactating dairy cows progressively decreased due primarily to two key reproductive parameters, low estrus detection and pregnancies per AI [12,13]. Current reports indicate that estrus detection rate is ~36% in lactating cows (MI DHIA 2014, yearly summary) and 70% in heifers. The dramatic change in estrus detection rate from the transition of heifer to cow, may be attributed to changes in circulating concentrations of estrogen and progesterone [1,3,5] as well as differences in environment, with cows spending more time on concrete [14,15] and being more susceptible to heat stress [16,17]. Fortunately, estrus detection rate (or service rate when referring to cows timed-inseminated) is significantly enhanced when cows are timed-inseminated with Ovsynch [18]. This is because Ovsynch can be utilized as a tool to control time to first AI and subsequent inseminations following a negative pregnancy diagnosis. Unfortunately, the second greatest obstacle, pregnancies per AI, is not enhanced with Ovsynch [18,19]. In the past 40 years, pregnancies per AI in lactating dairy cows decreased from around 65% in the 1950's [20,21] to approximately 35% [22,23] while pregnancies per AI in heifers remained steady at about 70% [19,24]. Ovsynch was developed to synchronize the time of ovulation to allow for timed-AI. Now, studies have focused on ways to

improve follicular and luteal dynamics during Ovsynch to improve pregnancies per AI of lactating dairy cows [11,25,26].

Three key physiological events transpire in fertility programs in a greater percentage of cows than in conventional synchronization programs: 1) A new estrous cycle and 2) subsequent ovulation of a first wave dominant follicle are induced within an 8-hour period. And, 3) complete luteolysis is controlled prior to endogenously induced luteolysis. These three events must occur to make these programs different than other synchronization programs.

This review focuses on specific pharmacological interventions utilizing only gonadotropin-releasing hormone (GnRH) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to manipulate ovarian development in lactating cows to generate physiological outcomes similar to nulliparous heifers and enhance fertility. These interventions manipulate antral age of the ovulatory follicle and the hormonal environment during its growth necessary to induce ovulation of a single competent oocyte. The outcomes of these interventions improve the chances of a pregnancy to a single AI compared to AI following estrus and are now referred to as Ovsynch-based "fertility programs for dairy cows." [11] They are distinguishable from other Ovsynch-based synchronization programs in the way follicles and corpora lutea are manipulated.

LIMITATIONS OF SOLELY USING OVSYNCH

Ovsynch is based on three pharmacological treatments [27]. The 1st treatment, GnRH, may induce an LH surge and may cause a mature functional dominant follicle(s) (DF) to ovulate [28]. In turn, ovulation of the DF induces subsequent emergence of a new follicular wave ~1.5 d later [27] followed by development of a new dominant follicle during the next 7 d. If a follicle does not respond to the GnRH it is likely the cow is in the first 3 to 4 d of a follicular wave [26].

During this early stage of a wave, the largest growing follicle may be too immature (e.g., no LH receptors [29]) to respond to the GnRH-induced LH surge caused by the 1st GnRH treatment [28]. If the DF is not responsive to a GnRH induced LH surge, it may develop into an ovulatory follicle during the remainder of the Ovsynch treatments or possibly become atretic prior to luteolysis [30]. If the follicle becomes atretic prior to $PGF_{2\alpha}$, a new follicular wave will emerge. The new DF from that wave will most likely not be mature enough 2 d later to respond to the LH surge induced by the final GnRH of Ovsynch. If the $PGF_{2\alpha}$ induces complete luteolysis, cows will likely display signs of estrus 3 to 4 d following AI following maturation of the newest pre-ovulatory follicle. This artifact is a common asynchrony of Ovsynch.

The second treatment, $PGF_{2\alpha}$, is administered to induce luteolysis, thus enabling the DF of the *new* follicular wave to develop into a pre-ovulatory follicle. Luteolysis following a single dose of $PGF_{2\alpha}$ may not be effective, particularly in multiparous cows. This will be discussed in more detail later in this paper. The third treatment, GnRH, is administered 56 to 60 h after $PGF_{2\alpha}$ [31] to induce a pre-ovulatory LH surge that triggers ovulation of the DF 24 to 32 h later [27]. This is highly effective in cows with functional pre-ovulatory follicles.

Cows treated with Ovsynch yield overall pregnancies per AI similar to those obtained after breeding to detected estrus (37 versus 39 %, respectively; $P > 0.10$) [18]. Up to 40% of cows may not have an ovulation synchronized with Ovsynch programs [25-27]. Non-synchronized cows will not be inseminated at an appropriate time relative to ovulation, or will not have luteolysis, thereby decreasing their chances of becoming pregnant [25,26]. Improving synchronization rates alone of Ovsynch could have a positive impact on reproductive performance. Vasconcelos et al. [25] attributed most of the variability in synchronization rate in cows to the stage of the estrous cycle in which Ovsynch was initiated. Cows started on Ovsynch

between d 5-9 of the estrous cycle had a greater probability of synchronizing and therefore had a greater chance of pregnancy [25,26].

DIFFERENCES BETWEEN FERTILITY AND SYNCHRONIZATION PROGRAMS

Studies indicate that synchronization rates can be significantly improved when lactating dairy cows are treated with a pre-synchronization program utilizing $PGF_{2\alpha}$ and GnRH compared to Ovsynch alone or Presynch-12 or -14/Ovsynch [26,32,33]. The key reason for greater synchrony was the synchronous initiation of a new estrous cycle, which allowed the first GnRH of Ovsynch to be administered near d 6 or 7 of the new cycle [25,26]. The data presented next will demonstrate that initiating the first GnRH of Ovsynch near d 6 or 7 of the estrous cycle enhances function of the ovulatory follicle and increases the percentage of cows that have ovulation to the final GnRH of Ovsynch [25,26]. This, in turn, allowed more cows the opportunity for pregnancy, and translated into increased pregnancies per AI [26].

CONTROL OF THE EMERGENCE OF THE OVULATORY FOLLICLE IS CRITICAL FOR OPTIMAL SYNCHRONIZATION

Attaining consistent ovulation in response to first GnRH of Ovsynch constitutes the first key step to optimizing synchronization of ovulation to Ovsynch in lactating dairy cows [25,26]. Ovulation to first GnRH of Ovsynch is followed by emergence of a new follicular wave, from which the ovulatory follicle of Ovsynch develops [27]. Thus, variation in response to first GnRH, leads to extreme variation in the timing of emergence of the ovulatory follicle of Ovsynch. This, in turn, results in substantial variation in size of ovulatory follicles at the time of

the final GnRH of Ovsynch [25,26]. This variation leads to a reduced chance of pregnancy [25,26].

The G6G program, for example, decreased variability in size of the ovulatory follicle and increased synchronization rate to Ovsynch [26]. In these experiments, cows were treated with 25 mg $PGF_{2\alpha}$ then 2 d later 100 μg GnRH. Then, cows received the first GnRH of Ovsynch either 4, 5, or 6 d later in Experiment 1[26], and either 6, 7 or 8 d later in Experiment 2 [34]. Controls in both experiments received only Ovsynch. Compared to Ovsynch alone, 6 d from the presynchronization treatment of GnRH until the first GnRH of Ovsynch significantly improved percentage of cows ovulating to first GnRH, percentage of cows responding to $PGF_{2\alpha}$ by luteolysis, and percentage of cows with both a luteolytic response to $PGF_{2\alpha}$ and ovulation to the final GnRH of Ovsynch. These improvements were repeated in Experiment 2 for d 6 compared to controls. In the two studies combined, ovulation rate to the first GnRH of Ovsynch averaged 90 % in the d 6 groups (n = 76). In addition, it appears that 7 d from presynchronization GnRH to the first GnRH of Ovsynch also improved these responses, particularly when cows initiated a new estrous cycle by responding to both presynchronization treatments. Thus, d 6 or 7 of the estrous cycle appear to be the ideal time of the estrous cycle to initiate Ovsynch to maximize ovulatory response to the first GnRH and luteolysis following $PGF_{2\alpha}$ [26,34].

Additional data reveal that cows ovulating in response to the first GnRH of Ovsynch yielded significantly less variability in pre-ovulatory follicle size at the final treatment of GnRH, a greater chance of luteolysis in response to the $PGF_{2\alpha}$ of Ovsynch, and a greater chance of ovulating to the final GnRH [25,26]. Also from this study [26], a positive linear relationship was detected between concentrations of estradiol at the final GnRH of Ovsynch, and the probability of pregnancy. In addition, a quadratic relationship was also detected between ovulatory follicle

size at final GnRH and the probability of a pregnancy. Cows with follicle sizes associated with a greater chance of pregnancy also had greater serum concentrations of estradiol. Thus, it is of critical importance to optimize the size of the ovulatory follicle to allow these follicles to secrete as much estradiol as possible at the time of the final treatment of GnRH of Ovsynch.

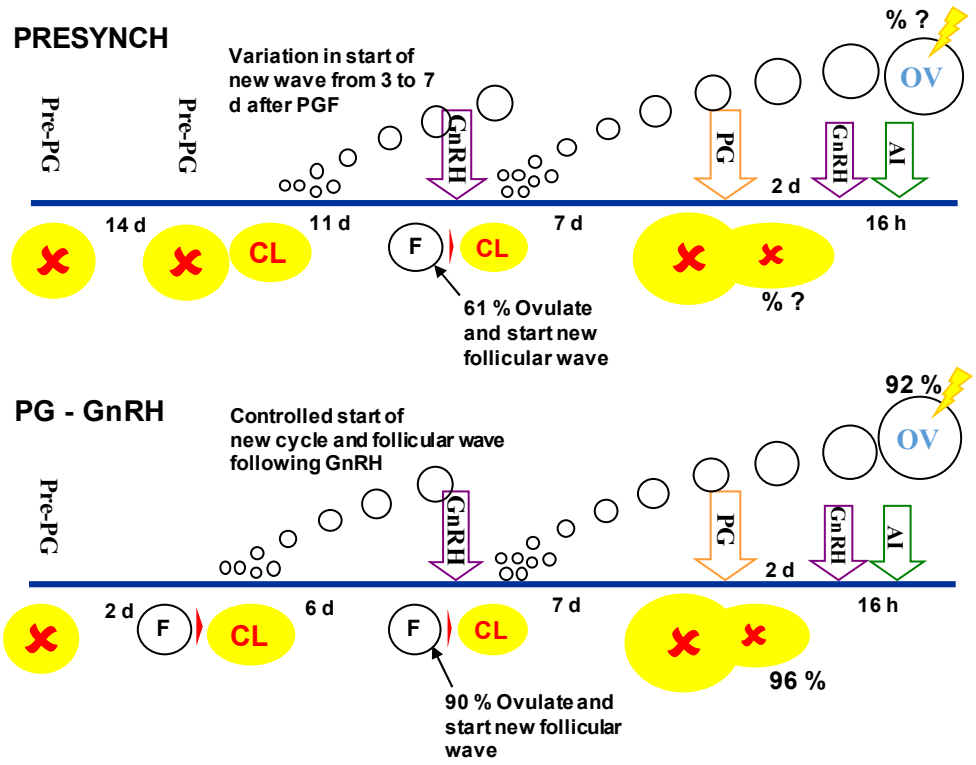

Figure 2.1. Description of potential difference in control of follicle development in Presynch versus the $PGF_{2\alpha}$ – GnRH presynchronization scheme proposed. Control of a new estrous cycle in more cows may allow the $PGF_{2\alpha}$ – GnRH scheme greater control of the ovulatory follicle following the first GnRH of Ovsynch.

These two experiments were designed to only test the impact of this presynchronization scheme on follicle and CL development in response to the Ovsynch treatments [26,34]. In these preliminary data, we show nearly a doubling of percent cows pregnant in the 6 or 7 d groups compared to Ovsynch alone. Figure 1 describes some of the potential differences between two

presynchronization schemes. Presynch utilizes two injections of $PGF_{2\alpha}$ 14 d apart and 11 to 14 d prior to the start of Ovsynch. Since $PGF_{2\alpha}$ only directly controls luteolysis, time to estrus and ovulation can be quite variable, as a result d of the cycle at the start of Ovsynch can be variable too. The likelihood of initiating ovulation to the first GnRH of Ovsynch in the 11 d interval is approximately 61% and 45% in the 14 d interval between second $PGF_{2\alpha}$ and first GnRH of Ovsynch [35]. In addition, cows treated with Ovsynch that are anovular, will not respond to the $PGF_{2\alpha}$ injections of Presynch, but will likely respond to the GnRH of the new proposed program, thus allowing the initiation of Ovsynch at the optimal time of a subsequent follicular wave.

If cows were on d 6 of the cycle at time of 1st GnRH of Ovsynch, 97% of cows induced *accessory* CL, had significantly greater P4 concentrations, and had a greater probability of a pregnancy [26,34]. Cows with both, a d 7 and 13 corpora lutea at time of $PGF_{2\alpha}$ of Ovsynch, have approximately 50 % greater progesterone concentrations compared to cows with only a d 13 corpus luteum [36]. Fertility programs enhance the percentage of cows that respond to the first GnRH of Ovsynch, and in turn, allows for more cows with accessory CL, greater concentrations of progesterone at time of induced luteolysis, and a greater chance for pregnancy.

ENHANCING CL REGRESSION

Circulating concentration of P4 during fertility treatments appears to be one of the most important markers of subsequent pregnancy success following timed-AI. In addition to the effect of levels of P4 prior to $PGF_{2\alpha}$ on fertility of lactating dairy cows previously discussed; serum concentrations of P4 following $PGF_{2\alpha}$ of Ovsynch has also been associated with P/AI of lactating dairy cows after timed-AI [37-39]. However, in this case, a slight increase on serum concentration of P4 appears to be detrimental for the success of timed-AI outcomes. Studies

using synchronization programs reported that cows with functional CL, that do not decrease circulating P4 to basal levels, have small to no chances of conceiving following timed-AI. In previous studies, probability of pregnancy decreased as circulating concentrations of P4 increased [37-39]. Time to reach complete luteolysis after $PGF_{2\alpha}$ of Ovsynch appears to also influence fertility since cows with a delay on P4 clearance had impaired fertility following timed-AI [39,40]. Therefore, it is essential that cows with functional CL have complete luteal regression following $PGF_{2\alpha}$ of Ovsynch, which is characterized as a drop of circulating P4 to basal levels prior to timed-AI. Reports of percentage of lactating dairy cows without complete luteolysis following $PGF_{2\alpha}$ of Ovsynch are between 5 and 30 % [37-39,41,42]. In addition, similar results were obtained using either of the two $PGF_{2\alpha}$ products available in the U.S.: dinoprost tromethamine (Lutalyse and ProstaMate) and cloprostenol sodium (Estrumate and estroPLAN). Although the mechanisms involved on the resistance of a mature d 7 or older CL to undergo complete luteolysis are not well characterized, some factors appear to influence the proportion of cows with complete luteolysis following $PGF_{2\alpha}$ of Ovsynch. A previous study from our laboratory identified that parity and service could affect percentage of cows with complete luteolysis [39]. A greater percentage of cows in first service underwent luteolysis compared to second and greater services (79 vs. 71%; respectively). Primiparous cows were also more likely to have complete luteolysis compared to multiparous cows (94 vs. 81%) [39]. This same study also reported that cows with greater circulating of P4 at time of $PGF_{2\alpha}$ of Ovsynch had a greater probability of complete luteal regression (Figure 2) [39]. This result was unexpected since cows with two CL, a mature and accessory CL, at time of $PGF_{2\alpha}$ of Ovsynch, have higher serum P4 at time of $PGF_{2\alpha}$ of Ovsynch, and were believed to have problems with luteolysis due the number of CL and the young age of the accessory CL (d 7).

In order to enhance percentage of cows with complete luteolysis following $PGF_{2\alpha}$ of Ovsynch, two different approaches have been tested: repeated administrations with $PGF_{2\alpha}$ commercial label dose [38] and increased label dose of $PGF_{2\alpha}$ administered once [43]. Percentage of cows with complete CL regression was increased when an additional $PGF_{2\alpha}$ treatment was administered 24 h after the $PGF_{2\alpha}$ of Ovsynch (95.6 %) compared to cows with only the $PGF_{2\alpha}$ of Ovsynch (84.6 %) [38]. Although there was an increase in percentage of cows with luteolysis, P/AI did not significantly increase in cows with two vs one $PGF_{2\alpha}$ treatment (44.7 vs. 41.5%) [38]. This difference of approximately 3 % in P/AI was already expected because only the extra 11 % of cows that had complete luteolysis (95.6 % - 84.6 %) in the two $PGF_{2\alpha}$ treatment benefited from the extra $PGF_{2\alpha}$. These 11 % extra cows would have approximately 40 % of pregnancy rate what would result in an extra ~ 4 % of increase in P/AI. In order to find statistical differences in such a small difference in proportions (binary variable), the study would need a greater number of subjects.

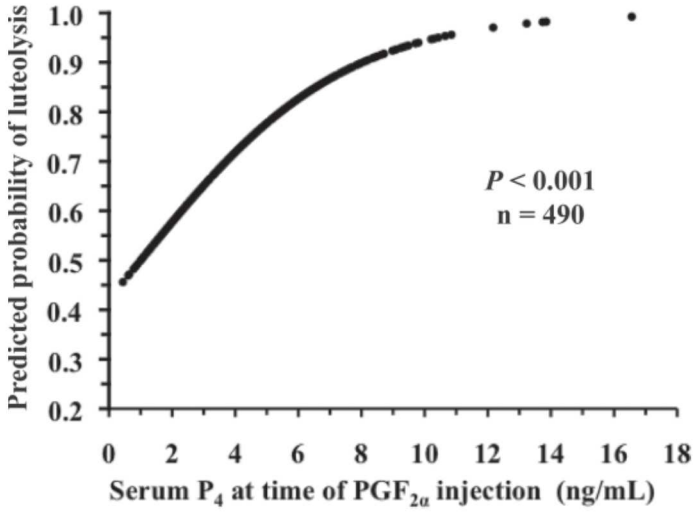

Figure 2.2. Predicted probability of complete luteolysis based on concentrations of progesterone (P4 < 0.5 ng/mL 56, 72, and 96 h after $PGF_{2\alpha}$ injection) at time of $PGF_{2\alpha}$ injection of Ovsynch in lactating dairy cows with functional corpus luteum (CL; P4 concentrations ≥0.24 ng/mL 24 h and ≥0.09 ng/mL 56 h after treatment; n = 490) at time of treatment. Published in Journal of Dairy Science (2011) [39].

Giordano et al. (2013) tested whether a 50% increase in the label dose (0.5 mg vs. 0.75 mg) of a $PGF_{2\alpha}$ analogue (cloprostenol) would increase the percentage of cows with complete luteolysis following $PGF_{2\alpha}$ of Ovsynch [43]. The greater dose of $PGF_{2\alpha}$ increased the percentage of cows with complete luteolysis (87.7 vs. 79.2 %), and tended to increase P/AI 39 d after AI in multiparous cows (45.4 vs. 40.9 %) [43]. However, it did not influence primiparous cows (92.8 vs. 89.7%) [43]. These studies indicated that some cows and/or their CL, are more resistant to complete luteolysis with the regular label dose of $PGF_{2\alpha}$. Taken together, there is compelling evidence indicating that insufficient luteolysis after $PGF_{2\alpha}$ of Ovsynch has a direct impact on reproductive performance of lactating dairy cow and that two administrations of $PGF_{2\alpha}$ 24 h apart can overcome this problem. Therefore, fertility treatments incorporated the use of second $PGF_{2\alpha}$ treatment 8 to 24 h after the $PGF_{2\alpha}$ of Ovsynch to enhance complete luteolysis rate and the chances of pregnancy following timed-AI.

SUMMARY

It is critical to induce ovulation either during the estrous cycle or in an anovular condition to generate a new follicular wave and an accessory corpus luteum. Ovulation rate following a GnRH-induced LH surge is greatest on d 6 or 7 of the estrous cycle, during the latter stages of the first follicular wave. It is also critical to initiate the induction of luteolysis of the spontaneous-formed CL as well as the accessory CL when the dominant follicle from the new follicular wave is at an ideal stage of maturity. Induction of complete luteolysis of these corpora lutea is critical and cannot be jeopardized; therefore, it is imperative to utilize two doses of $PGF_{2\alpha}$ 8 to 24 h apart. In essence, inducing the initiation of a new wave, and causing ovulation of the DF from that new wave, manipulates the age of the ovulatory follicle similar to that of heifers

during an estrous cycle [3-5]. Presynch-10 or 11, G6G, and Double Ovsynch create these physiological differences at a greater rate compared to Ovsynch alone and Presynch 12 or 14, and may increase pregnancies per AI outcomes 30 to 60 % [26,32,35,44-46].

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CHAPTER 3

ENHANCING ENDOGENOUS PROGESTERONE DURING GROWTH OF THE OVULATORY FOLLICLE IS POSITIVELY ASSOCIATED WITH FERTILITY OF DAIRY COWS TREATED WITH PRESYNCH-11/OVSYNCH, DOUBLE OVSYNCH, AND G6G/OVSYNCH

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ABSTRACT

Pre-synchronization strategies that enhance ovulation to first GnRH of Ovsynch have a greater chance of enhancing progesterone during ovulatory follicle growth. The objectives of this study were to 1) determine the effect of three pre-synchronization strategies on pregnancy/AI (P/AI) of lactating dairy cows, 2) determine the relationship between P4 at PGF of Ovsynch on P/AI, and 3) determine if decreasing time from follicular wave induction to luteolysis during Ovsynch enhances fertility. Cows (n=2453) were assigned to four treatments by parity to receive Presynch-11 (P11), G6G/Ovsynch (G6G), and Double Ovsynch 7 or 5 d between G and PGF of Ovsynch (DO7, DO5) beginning 39 to 51 DIM. Cows received final GnRH of Ovsynch 72 h after PGF and AI 16 h later. Ovulation was diagnosed with ultrasound. Serum was collected at strategic intervals to assay P4. Pregnancy was diagnosed with ultrasound 32 d post-AI. Percent cows with P4 >1 ng/mL were different between treatments at final PGF of the Presynch program (65, 56, 78, 77%; P<0.01, n=2451), at first GnRH of Ovsynch (67, 76, 87, 87%; P<0.001, n=2453), and at PGF of Ovsynch (84, 86, 93, 93%; P<0.001, n=2451) for P11, G6G, DO7 and DO5. Ovulation rate in response to the first GnRH of Ovsynch (80, 85, 89, and 86%; P<0.05, n=1878), percent cows with luteolysis after final PGF of Ovsynch (96, 92, 93, and 43%; P<0.001, n=2223) were different for P11, G6G, DO7 and DO5. PAI in cows (n=1802) that ovulated to first GnRH of Ovsynch and had P4 >1ng/mL at PGF and decreased to < 0.3 ng/mL P4 72h later were 52, 50, 49, and 48% (P=0.7) for P11, G6G, DO7 and DO5. Concentrations of P4 at time of PGF injection was positively associated with pregnancy outcome: 33, 43, 44, 50, 51 and 57% PAI for P4 ranges of 1-2 (n=171), 2-3 (n=136), 3-4 (n=201), 4-5 (n=308), 5-6 (n=327) and >6 (n=659) ng/mL P4 (P<0.05 for P4 < 4ng/mL vs. P4 > 4 ng/mL). Enhancing progesterone

during growth of the ovulatory follicle is associated with enhanced PAI of lactating dairy cows. Decreasing time from wave induction to luteolysis did not increase fertility.

INTRODUCTION

Time to artificial insemination (AI) was limited by estrus detection. Synchronization of ovulation strategies utilizing gonadotropin releasing hormone (GnRH) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) through Ovsynch technology (GnRH – 7 d - $PGF_{2\alpha}$ – 56 h – GnRH – 16 h AI allows for timed-AI, and thus greater control of service rate and days open [1,2]. During the last 20 years following the development of Ovsynch, research have identified limiting factors of the Ovsynch technology, which resulted in several improvements of the original protocol [1,3]. One of the critical factors identified was that pregnancy success after timed-AI is optimized when Ovsynch is initiated with the presence of a CL (in diestrus) [4-6]. Another critical point is the positive response to all three treatments of Ovsynch; which includes ovulation to the first GnRH [7-9], complete corpus luteum (CL) regression after the $PGF_{2\alpha}$ [10,11], and ovulation after final GnRH and following timed-AI [7,9,12]. In this scenario, Ovsynch has maximal control of the hormone environment, mainly progesterone (P4), and the antral age of the ovulatory follicle [13]. To achieve this optimal scenario, days 6 or 7 were established as the best days of the estrous cycle to initiate Ovsynch [7,12]. Three pre-synchronization strategies were develop to increase percentage of cows on d 6 or 7 of the cycle: G6G [12], Double-Ovsynch [14] and Presynch-11 or -10 [15].

Presynch-11 utilizes 2 treatments of $PGF_{2\alpha}$ 14 d apart and 11 d prior to Ovsynch [15]. The majority of cycling cows that respond to the second $PGF_{2\alpha}$ will be detected in estrus 3 to 4 d later [16,17], initiating Ovsynch on d 7 or 8 of the estrous cycle. However, anovular cows do not seem

to benefit from presynchronization protocols that only use $PGF_{2\alpha}$ -induced luteolysis since $PGF_{2\alpha}$ does not induce ovulation and formation of a new CL in cows without a CL [18]. In the other hand, G6G uses $PGF_{2\alpha}$ and GnRH 2 d apart to initiate a new estrous cycle 6 d prior to Ovsynch [12]. Studies from our laboratory indicated that approximately 70 % of cows in a random stage of the cycle respond to both treatments [9,12]. Ovulation to the first GnRH of Ovsynch following G6G was 64 to 85 % [12,19,20], and it was greater compared to Ovsynch initiated in a random period of the estrous cycle [12]. Double Ovsynch adds another GnRH treatment 7 d prior to G6G, forming an entire Ovsynch protocol (GnRH – 7 d – $PGF_{2\alpha}$ – 2 d – GnRH) used for pre-synchronization and to initiate a new estrous cycle 7 d prior the final timed-AI Ovsynch [14]. One of the benefits of using Ovsynch for pre-synchronization is the increase in the percentage of cows cycling prior to the timed-AI Ovsynch. It has been demonstrated that Ovsynch can successfully resume cyclicity in anovular cows. Ovulation to first GnRH of Ovsynch following Double-Ovsynch pre-synchronization was 72 to 80 % [14,21].

Currently, P/AI for first service cows inseminated following these programs have been reported to be greater compared to cows inseminated after estrus [22,23]. However, to our knowledge, prior to the initiation of this experiment, there was no study that directly compared reproductive parameters of fertility treatments in high producing lactating dairy cows. In addition, it was not known if a reduction in the duration of the dominance period of the ovulatory follicle by reducing the length of Ovsynch to 5 d could improve P/AI for cows submitted to a fertility program.

Therefore, this experiment focused on comparisons of pre-synchronization strategies that allowed for the initiation of Ovsynch on d 6 of the cycle. Treatments test pre-synchronization programs with and without GnRH treatment. The objectives of the present study were: (1)

determine differences in P/AI between treatments, following first service in lactating dairy cows; (2) determine difference in ovulation rates to first GnRH of Ovsynch between treatments; and (3) determine the relationship between concentrations of progesterone at time of $PGF_{2\alpha}$ of Ovsynch and pregnancy/AI of lactating dairy cows. We hypothesized that: (1) a pre-synchrony strategy utilizing $PGF_{2\alpha}$ and GnRH will enhance P/AI of lactating dairy cows compared to a pre-synchrony strategy using only $PGF_{2\alpha}$; (2) GnRH 7 d prior to the pre-synchrony $PGF_{2\alpha}$ of G6G will enhance P/AI in lactating dairy cows; and (3) reducing time from first GnRH of Ovsynch to $PGF_{2\alpha}$ 2 d will enhance P/AI of lactating dairy cows treated with a modified Double-Ovsynch program.

MATERIALS AND METHODS

This study was conducted between October 2009 and May 2010 in two farms owned by the same person (De Saegher Dairy). Herd of primiparous cows were located in Elsie (MI), and multiparous cows were located in Middleton (MI). Both farms had similar herd management. Cows were housed in free stall barns with free access to water and fed a TMR 2x/d, which consisted of corn and alfalfa silages and corn-soybean meal-based concentrates. TMR was formulated following recommendations for lactating dairy cows (NRC, 2001). Cows were milked 3 times daily. Herd milk production during study period averaged 33 kg/cow/d.

Trained personnel from our laboratory administered all treatments of $PGF_{2\alpha}$ (500 mg cloprostenol; 2 mL of Estrumate, Merck Animal Health) and GnRH (86 µg gonadorelin acetate, 2 mL of Fertagyl, Merck Animal Helath) using single dose syringes in semimembranosus or semitendinosus muscles of cows. The Institutional Animal Care and Use Committee at Michigan State University approved all animal handling and procedures described in this manuscript.

Experimental design

Weekly groups of lactating dairy cows (Total n = 2,488) were blocked by parity category and assigned randomly to one of the four treatments: control (Presynch-11), G6G, Double-Ovsynch-7d (DO7) and Double-Ovsynch-5d (DO5). Cows assigned to control initiate Presynch-11 (P11) between 42 and 48 DIM, which consisted of 2 treatments of $PGF_{2\alpha}$ 14 d apart with the second injection 11 d prior to the first GnRH of Ovsynch. Cows treated with G6G started treatment between 49 and 55 DIM with $PGF_{2\alpha}$ followed in 2 d with GnRH, then 6 d later Ovsynch was initiated. Cows in DO7 initiated treatment between 52 and 58 DIM with a pre-synchronization Ovsynch (GnRH – 7 d – $PGF_{2\alpha}$ – 2 d – GnRH; Figure 1) ending 6 d prior to the first GnRH of Ovsynch for timed-AI. Cows in P11, G6G and DO7 received the same Ovsynch for timed-AI (GnRH – 7 d – $PGF_{2\alpha}$ – 8 h – $PGF_{2\alpha}$ – 64 h – GnRH – AI; Figure 1). Cows treated with DO5 also used an Ovsynch for pre-synchronization that was completed 6 d prior to the initiation of the Ovsynch for timed-AI. Cows in DO5 received a shorter Ovsynch protocol for timed-AI compared to the other treatments (GnRH – 5 d – $PGF_{2\alpha}$ – 8 h – $PGF_{2\alpha}$ – 64 h – GnRH – AI; Figure 1). To enhance percentage of cows with complete luteolysis, all cows in the experiment received a second $PGF_{2\alpha}$ 8 h following the first $PGF_{2\alpha}$ of Ovsynch. Timing of final GnRH of Ovsynch was 72 h following the first $PGF_{2\alpha}$-induced luteolysis of timed-AI Ovsynch. A subset of cows (n = 317) was detected in estrus on day of last GnRH of Ovsynch and received AI only the same day (n = 55), or on both days, day of GnRH and day of timed-AI (n = 262). All the other cows received only timed-AI 16 h after the final GnRH of Ovsynch between 75 and 88 DIM. Seven AI technicians blind to treatments performed artificial insemination. High fertility service sires (n=5) were assigned randomly to cows by treatments within blocks by bull

collection batch 1 and 2. Cows subsequently identified as sick or that have left the herd between treatment assignment and pregnancy diagnosis were not included in the results.

Ovarian ultrasonography and pregnancy diagnoses

Ovaries were scanned in a random subset of cows on the day of first GnRH of timed-AI Ovsynch and on the day of final GnRH of timed-AI Ovsynch (Figure 1). To determine ovulation, cows' ovaries were also scanned 2 d after the first GnRH of Ovsynch. Ultrasonography examination of ovaries was conducted by three skilled technicians from our laboratory blind to treatment using an Aloka SSD-900 ultrasound machine with a 7.5 MHz linear array transducer (Aloka Co. Ltd., Wallingford, CT), and a Sonosite MicroMaxx ultrasound machine with a 5-10 MHz multi-frequency linear array transducer. Average diameter of follicles ≥ 9 mm and CL were estimated using on-screen side grids of 10 mm of length. Ovulation following first GnRH of Ovsynch was determined by the disappearance of one dominant or co-dominant follicles (≥ 10 mm), previously visualized at the d of GnRH treatment and the presence of a new CL 2 d later. Farm veterinarians (Dr. Michael Trombley and Dr. Russ Seifferlein from Clinton Veterinary Services, PC., St Johns, MI) performed pregnancy diagnosis at 32 d post-AI using ultrasound. Pregnancy was confirmed by presence of an embryo with heartbeat.

Blood collection and hormonal assays

Blood samples were collected via coccygeal venipuncture using vacutainer tubes without anticoagulant (BD Vacutainer, Franklin Lakes, NJ) on d of $PGF_{2\alpha}$ of each presynchronization prior to Ovsynch (n = 2,434) and 2 d later (n = 2,397), on d of each injection of timed-AI Ovsynch (first GnRH, n = 2,437; $PGF_{2\alpha}$, n = 2,428; and final GnRH, n = 2,410) and at d of

timed-AI (n = 2,398). Samples were refrigerated, transported to our laboratory, and maintained in a refrigerator at 4 °C overnight. Blood samples were centrifuged at 2000 x g for 20 min at 4 °C to separate serum. Serum was stored at -20 °C for later hormonal analyses. Serum concentrations of P4 were quantified in all samples via RIA (Coat-a-Count, Siemens Diagnostic, Los Angeles, CA). Intra- and inter-assay CV were < 10 %.

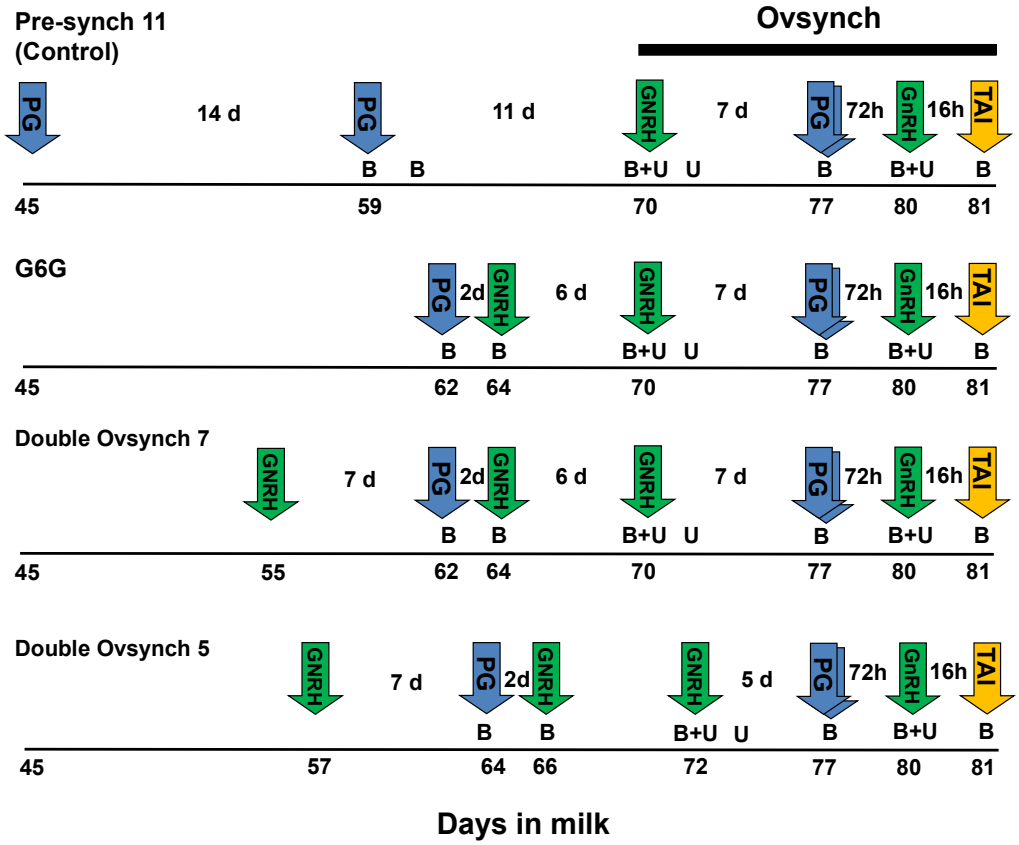

Figure 3.1. Schematic illustration of experimental design with time of hormone injections ($PGF_{2\alpha}$ and GnRH), blood collection (B) and ultrasonography examination of ovaries (U) for each treatment, Pre-synch-11 (Control) vs. G6G vs. Double Ovsynch 7 (DO7) vs. Double Ovsynch 5 (DO5). PG= $PGF_{2\alpha}$; TAI = timed artificial insemination.

Analysis of luteal function

Cows with serum concentrations of P4 ≥ 1.00 ng/mL were considered to have a functional CL. Cows were considered to be cycling prior to or on the day of the first GnRH of timed-AI

Ovsynch when serum P4 was ≥ 1.00 ng/mL in at least one of the three collections times prior to $PGF_{2\alpha}$ of the timed-AI Ovsynch. Cows with P4 < 0.50 ng/mL 2 d after $PGF_{2\alpha}$ of the presynchronization prior to Ovsynch considered to have complete CL regression. Cows were only considered to undergone complete luteolysis after the $PGF_{2\alpha}$ of Ovsynch when P4 was < 0.30 ng/mL 72 and 88 h after this $PGF_{2\alpha}$. Only cows with functional CL on $PGF_{2\alpha}$ days were considered for luteolysis analysis. Cows were considered to start a new cycle after presynchronization when complete luteolysis (P4 < 0.50 ng/mL) was achieved, 2 d after $PGF_{2\alpha}$ of the presynchronization and a functional CL (P4 ≥ 1.00 ng/mL) was present at time of first GnRH of Ovsynch.

Statistical analyses

Calculation of required total sample size for the present experiment was performed in G*Power software [24] using the z test for logistic regression. Input parameters used were: α = 0.05, β = 0.85, two-tail, binomial distribution and odds ratio of 1.28. Power analysis indicated that a total of 2,411 subjects were needed to detect a 6 % point difference in P/AI (40 vs. 46 %) between treatments.

Binomial variables were analyzed using logistic regression with a generalized linear mixed model implemented with the GLIMMIX procedure of SAS (Version 9.4, SAS Inst., Inc., Cary, NC). The model considered treatments (P11, G6G, DO7 and DO5) and parity category (first, second and third or later) as fixed effects. Two-way interactions treatment by parity category was also included in the model.

Continuous variables such as concentrations of P4 and ovulatory follicle size were analyzed using a linear mixed linear model applying the MIXED procedure of SAS. The model

used treatments (P11, G6G, DO7 and DO5) and parity category (first, second and third or later) as fixed effects. Two-way interactions treatment by parity category was also included in the model. Normal distribution of the residuals was tested with studentized residual plots for each variable. Residuals were considered normally distributed for all variables. Means and probabilities values were considered different when < 0.05 and tendency for difference when < 0.10.

Verification of balance of parity number and DIM at AI between treatments

Parity (P = 0.65) number were balanced between treatments. Mean ± SEM parity number for all cows was 1.86 ± 0.02, ranging from 1 to 8. Days in milk at AI also did not differ (P = 0.64) between treatments. Overall mean ± SEM for DIM at AI was 81.4 ± 0.1, ranging from 75 to 88 DIM. Therefore, parity number and/or DIM at AI did not confound treatment effects.

RESULTS

Effect of treatment and parity on pre-synchronization outcomes based on luteal function

Treatments DO7 (77.7 %) and DO5 (77.4 %) increased (P < 0.001) the percentage of cows with functional CL at time of presynchronization $PGF_{2\alpha}$ compared to Control P11 (65.0 %) and G6G (55.5 %; Figure 6.2). The first $PGF_{2\alpha}$ of Control P11 improved (P < 0.001) the percentage of cows with functional CL at time of the second $PGF_{2\alpha}$, compared to cows in random stages of the cycle at $PGF_{2\alpha}$ of G6G pre-synchronization (55.5 %; Figure 6.2). Cows with functional CL in random stages of the estrous cycle at $PGF_{2\alpha}$ of G6G presynchronization had greater (P = 0.048) mean concentration of P4 compared to cows treated with DO7 and DO5 at $PGF_{2\alpha}$ of presynchronization (Figure 6.3). Cows treated with P11 had greater mean serum P4

at $PGF_{2\alpha}$ of presynchronization compared to cows treated with DO7 (Figure 6.3). There was no effect (P = 0.42) of treatment on percentage of cows with complete luteolysis after presynchronization $PGF_{2\alpha}$ (P11, 81.2 %; G6G, 78.2%, DO7, 81.2 %; DO5, 81.4 %).

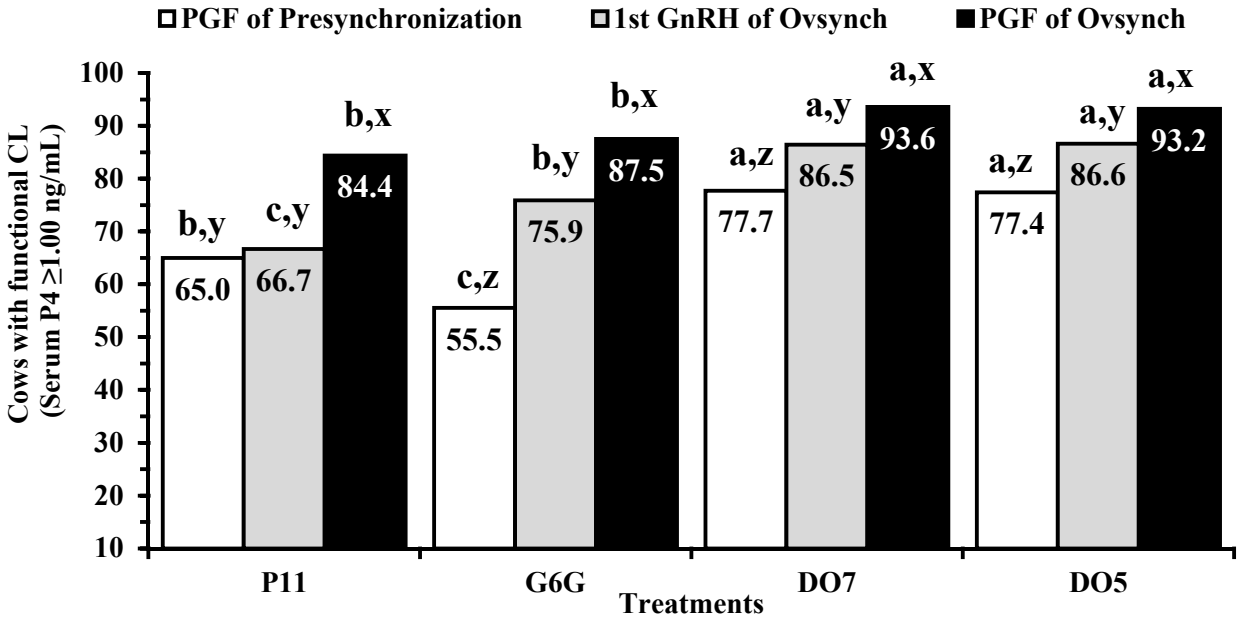

Figure 3.2. Effect of treatment on cows with functional CL* at $PGF_{2\alpha}$ of each pre-synchrony strategy (2nd of Presynch-11) and at $PGF_{2\alpha}$ of Ovsynch in lactating dairy cows (P<0.01; n=2474). *Functional CL = >1 ng/mL P4. Different letters (a,b,c) show a statistically significant difference (P<0.01) between treatments within $PGF_{2\alpha}$ times. Different letters (x,y,z) show a statistically significant difference (P<0.01) between $PGF_{2\alpha}$ times within treatments.

A smaller (P < 0.001) percentage of first parity cows (58.9 %) had functional CL at $PGF_{2\alpha}$ of presynchronization compared to second (75.1 %) and later parity cows (78.8 %). There was no interaction between treatment and parity category (P = 0.55). Parity category also had an effect (P < 0.001) on mean serum P4 for cows with functional CL. Mean serum P4 enhanced as parity category increased (first: 4.18 ± 0.09 ng/mL vs. second: 4.53 ± 0.11 ng/mL vs. third and greater: 4.99 ± 0.11 ng/mL). A greater (P < 0.001) percentage of first parity cows (85.3 %) had complete luteolysis compared to second (75.3 %), and later parity cows (73.9 %).

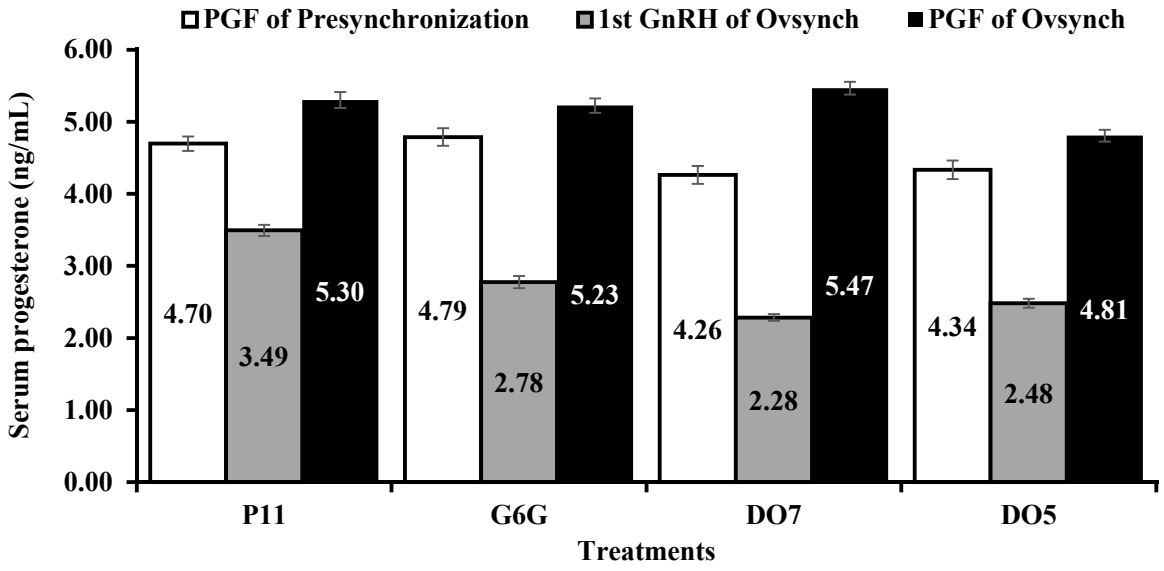

Figure 3.3. Effect of treatment on circulating concentrations of progesterone at $PGF_{2\alpha}$ of pre-synchronization strategy (2nd of Presynch-11), first GnRH of Ovsynch and at $PGF_{2\alpha}$ of Ovsynch in lactating dairy cows (P<0.01; n=2474). Only cows with functional CL were included in this analysis.

Treatments DO7 (95.1 %) and DO5 (95.5 %) increased ($P < 0.02$) the percentage of cows considered to be cycling prior to the first GnRH of Ovsynch compared to G6G (87.2 %). Control P11 (77.6 %) had a smaller ($P < 0.001$) percentage of cows considered to be cycling compared to the other treatments. An effect of parity was also detected in cycling status ($P < 0.001$). A smaller ($P < 0.001$) percentage of first parity cows (82.4 %) was cycling compared to second parity (92.7 %) and later parity cows (95.6 %). In addition, percentage of cows cycling had a trend ($P = 0.07$) to be greater in second parity compared to third or later parity cows. Interaction treatment and parity number was not significant ($P = 0.38$) for cycling status.

A greater ($P < 0.001$) percentage of cows treated with DO7 (71.3 %) or DO5 (70.8 %) started a new cycle following pre-synchronization, compared to the G6G (58.0 %) and P11 (52.6 %). Treatment G6G increased ($P = 0.04$) the proportion of cows starting a new cycle after pre-synchronization compared to P11. Third and later parity cows (57.4 %) had a smaller ($P < 0.05$)

percentage of success to start a new cycle after pre-synchronization, compared to first (64.9 %) and second parity cows (62.9 %). There was no interaction treatment by parity category (P = 0.54).

Effect of treatment and parity on luteal function at first GnRH of Ovsynch

Treatments DO7 and DO5 increased the percentage of cows with a functional CL at time of first GnRH of Ovsynch compared to G6G and P11 (Figure 6.2). Treatment P11 had the smaller percentage of cows with functional CL at time of first GnRH of Ovsynch compared to cows treated with G6G (Figure 6.2). Considering only cows with functional CL at time of the first GnRH of Ovsynch, treatment P11 increased (P < 0.001) mean serum P4 compared to the other treatments (Figure 6.3). Cows treated with G6G also had greater (P < 0.001) mean serum P4 at first GnRH of Ovsynch compared to DO7 and DO5 (Figure 6.3).

Parity category also had an effect (P = 0.02) on percentage of cows with a functional CL at time of first GnRH of Ovsynch. A greater (P < 0.02) percentage of second parity cows (82.3 %) had a functional CL compared to first parity (76.1 %) and third or greater parity cows (77.9 %). When considering only cows with functional CL at time of first GnRH of Ovsynch, third or greater parity cows (2.66 ± 0.08 ng/mL) had lower (P = 0.047) mean circulating P4 compared to first parity cows (2.78 ± 0.05 ng/mL) but similar (P = 0.24) mean serum P4 compared to second parity cows (2.77 ± 0.07 ng/mL).

Effect of treatment and parity on ovulatory response to the first GnRH of Ovsynch

Control P11 (80.1 %) had a smaller ($P \leq 0.03$) percentage of cows with ovulation after first GnRH of Ovsynch compared to G6G (85.1 %), DO7 (89.7 %) and DO5 (86.3 %; Figure 6.4). A greater (P = 0.02) percentage of cows treated with DO7 ovulated to the first GnRH of

Ovsynch compared to G6G. There was no effect ($P \leq 0.84$) of parity category on percentage of cows with ovulatory response to the first GnRH of Ovsynch.

Overall double ovulation rate for the first GnRH of Ovsynch was 7.8 %. Treatment did not influence ($P = 0.45$) the percentage of cows with double ovulations on the first GnRH of Ovsynch. However, an interaction between treatment and parity category was observed ($P < 0.01$). Third or later parity cows treated with DO7 (13.4 %) and DO5 (17.8 %) had greater ($P \leq 0.01$) double ovulation rate compared to cows in first parity DO7 (5.1 %) and DO5 (3.2 %).

Pre-ovulatory follicle size at first GnRH of Ovsynch for control cows was greater ($P < 0.001$) for control P11 (16.9 ± 0.2 mm) cows compared to G6G (15.2 ± 0.2 mm), DO7 (15.3 ± 0.2 mm), and DO5 (15.2 ± 0.2 mm). First parity cows (15.1 ± 0.1 mm) had smaller ($P < 0.001$) pre-ovulatory follicles compared to second (16.1± 0.2 mm) and later parity cows (16.4 ± 0.2 mm).

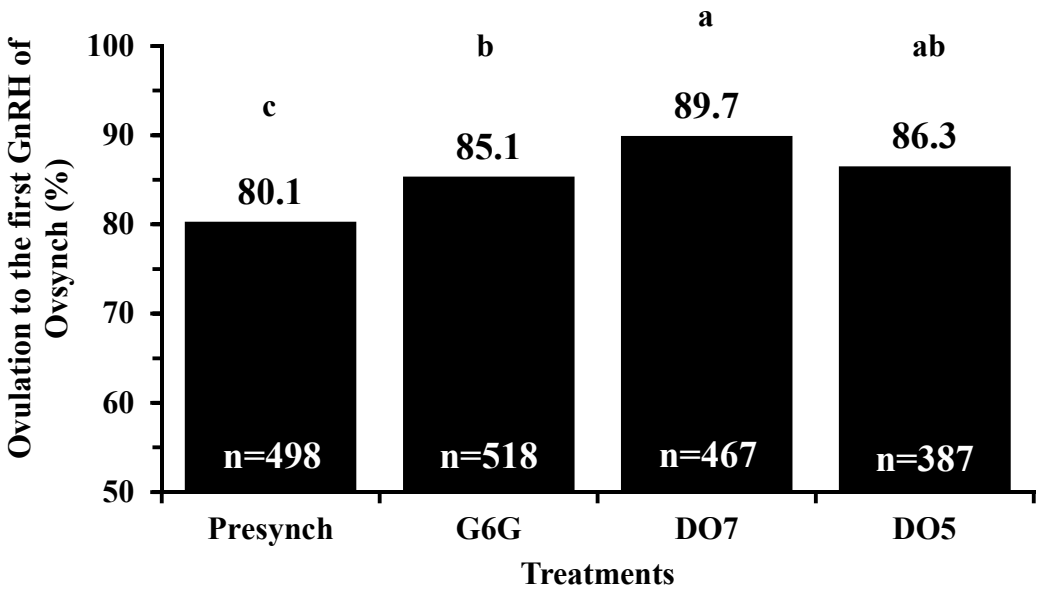

Figure 3.4. Effect of treatment on percent of cows that ovulated 2 d following first GnRH of Ovsynch in lactating dairy cows (P<0.01; n=1870). Different letters (a, b, c) show a statistically significant difference (P<0.01) between treatments.

Effect of treatment and parity on luteal function at $PGF_{2\alpha}$ of Ovsynch

Percentage of cows with functional CL at $PGF_{2\alpha}$ of Ovsynch was reduced ($P \leq 0.02$) for Control P11 (84.4 %) compared to G6G (87.5 %), DO7 (93.2 %) and DO5 (93.6 %; Figure 6.2). Treatments DO7 and DO5 had a tendency ($P \leq 0.09$) to increase percentage of cows with functional CL at $PGF_{2\alpha}$ of Ovsynch compared to G6G. Percentage of cows with functional CL at $PGF_{2\alpha}$ of Ovsynch was smaller ($P \leq 0.02$) for first parity cows (86.5 %) compared to second (91.3 %) and later parity cows (92.9 %). The interaction treatment by parity category was not significant ($P = 0.21$).

For cows with functional CL, cows treated with DO5 (4.81 ± 0.08 ng/mL) had lower ($P < 0.01$) mean serum P4 compared to Control P11 (5.30 ± 0.11 ng/mL), G6G (5.23 ± 0.10 ng/mL) and DO7 (5.47 ± 0.09 ng/mL). Second parity cows (5.47 ± 0.09 ng/mL) had greater ($P < 0.01$) mean serum P4 compared to first (5.14 ± 0.07 ng/mL) and third or later parity cows (5.13 ± 0.09 ng/mL). No interaction between treatment by parity category was found ($P = 0.37$).

Effect of treatment and parity on luteolysis response after $PGF_{2\alpha}$ of Ovsynch

DO5 treatment (39.6 %) decreased ($P < 0.001$) the percentage of cows with complete luteolysis after $PGF_{2\alpha}$ of Ovsynch compared to Control P11 (94.9 %), G6G (90.7 %), and DO7 (92.5 %; Figure 6.5). A greater ($P = 0.01$) percentage of cows treated with Control P11 underwent complete CL regression after $PGF_{2\alpha}$ of Ovsynch compared to cows treated with G6G. A greater ($P < 0.01$) percentage of first parity cows (84.9 %) had complete luteolysis after $PGF_{2\alpha}$ of Ovsynch compared to second parity (79.6 %) and third or later parity (75.8 %). No interaction between treatment and parity category was detected ($P = 0.92$).

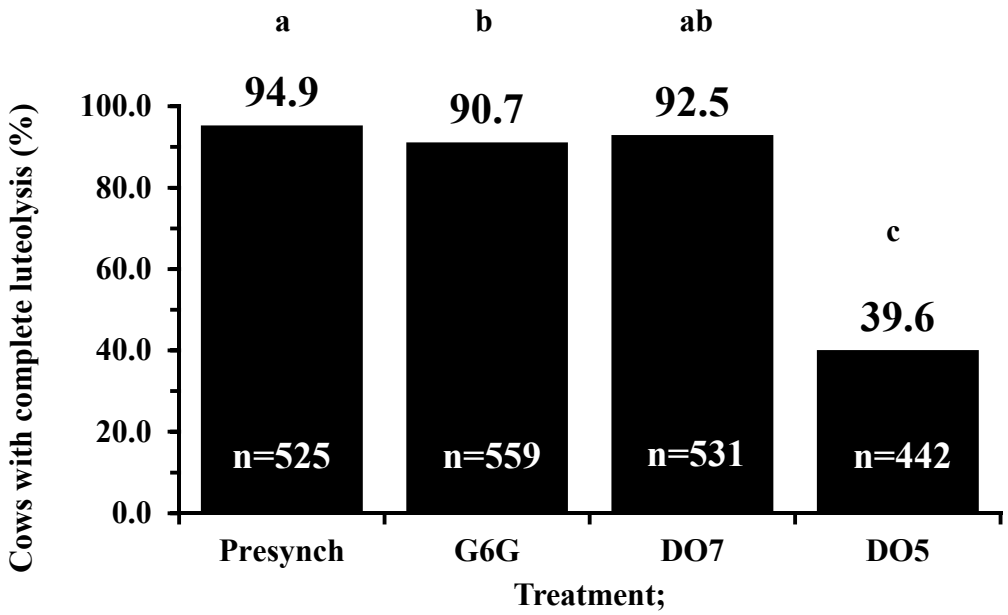

Figure 3.5. Effect of treatment on percent cows with complete luteolysis in lactating dairy cows (P<0.01; n=2223) following $PGF_{2\alpha}$ of Ovsynch. Complete luteolysis defined as circulating concentrations of progesterone <0.3 ng/mL 72 h post-initial PG. Different letters (a, b, c) show a statistically significant difference (P<0.01) between treatments.

Effect of treatment and parity on percentage of cows observed in estrus on the day of last GnRH of Ovsynch

A greater ($P < 0.01$) percentage of cows treated with P11 (17.3 %) and G6G (16.1 %) were detected in estrus on the day of final GnRH of Ovsynch compared to DO7 (10.8 %). A smaller ($P < 0.001$) percentage of cows treated with DO5 (4.2 %) was observed in estrus compared to P11, G6G and DO7. An effect of parity was also observed ($P < 0.001$) for cows detected in estrus on d of last GnRH of Ovsynch. Third or later parities cows (21.6 %) had a greater ($P < 0.001$) percentage of cows detected in estrus on the day of last GnRH of Ovsynch compared to first parity (8.6 %) and second parity cows (12.3 %). Percentage of cows in estrus was also greater ($P = 0.03$) for second parity cows compared to first parity cows. Interaction treatment and parity category was not observed ($P = 0.29$).

Cows observed in estrus had greater ($P < 0.001$) P/AI 32 d compared to cows that were not detected in estrus (53.3 vs. 38.7 %; respectively). Interaction between treatment and detection of estrus was no observed ($P = 0.77$) for P/AI, indicating a similar positive effect of estrus on P/AI across treatment. Interaction between parity category and detection of estrus was also not found ($P = 0.13$), demonstrating that cows from different parity categories had a similar positive influence of estrus on P/AI.

Effect of treatment, parity and luteolysis for the last $PGF_{2\alpha}$ of Ovsynch on P/AI 32 d post-AI

Cows treated with DO5 had reduced P/AI 23 d post-AI compared to cows treated with the other treatments (Figure 6.6). In addition, first parity cows (42.2 %) had greater ($P = 0.02$) P/AI compared to third or later parities (38.3 %). There was no interaction between treatment by parity category ($P = 0.11$). Cows with complete luteolysis (50.4 %) following last $PGF_{2\alpha}$ of Ovsynch had greater ($P < 0.001$) P/AI compared to cows without complete luteolysis (14.0 %). When only cows with complete luteolysis were analyzed, P/AI did not differ between treatments (Figure 6.7). A strong positive relationship was found between circulating concentrations of P4 and P/AI in lactating dairy cows, with or without complete luteolysis for the last $PGF_{2\alpha}$ of Ovsynch (Figure 6.8).

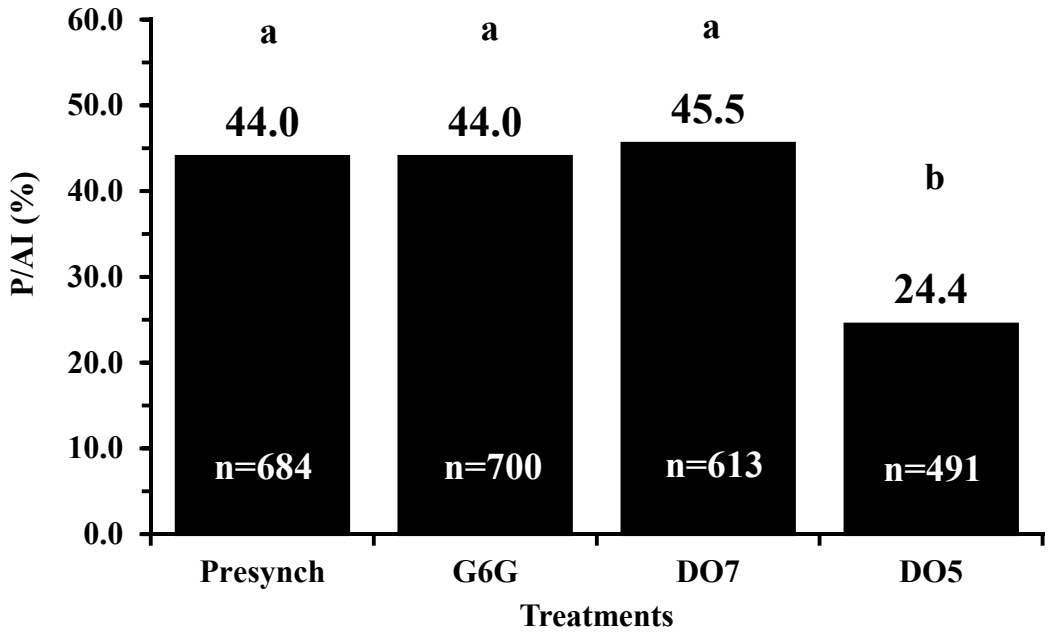

Figure 3.6. Effect of treatment on pregnancy/AI in lactating dairy cows (P<0.01; n=2488). *No effect of parity (P=0.06) or parity x treatment interaction (P=0.8). Different letters (a, b) show a statistically significant difference (P<0.01) between treatments.

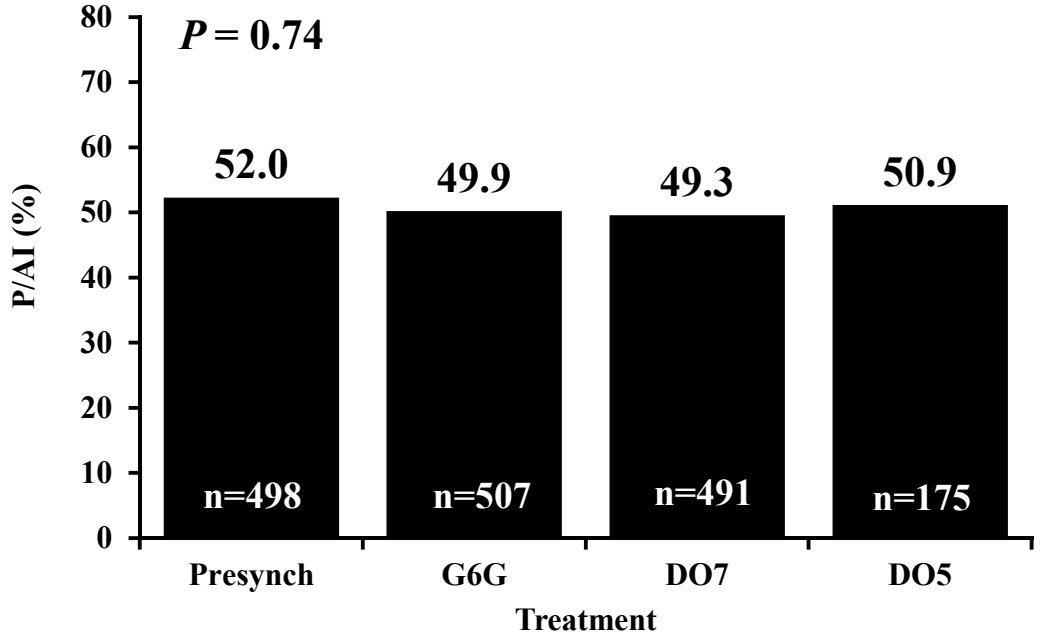

Figure 3.7. Effect of treatment on pregnancy / AI in cows with luteolysis following $PGF_{2\alpha}$ of Ovsynch in lactating dairy cows (P= 0.74; n=1671).

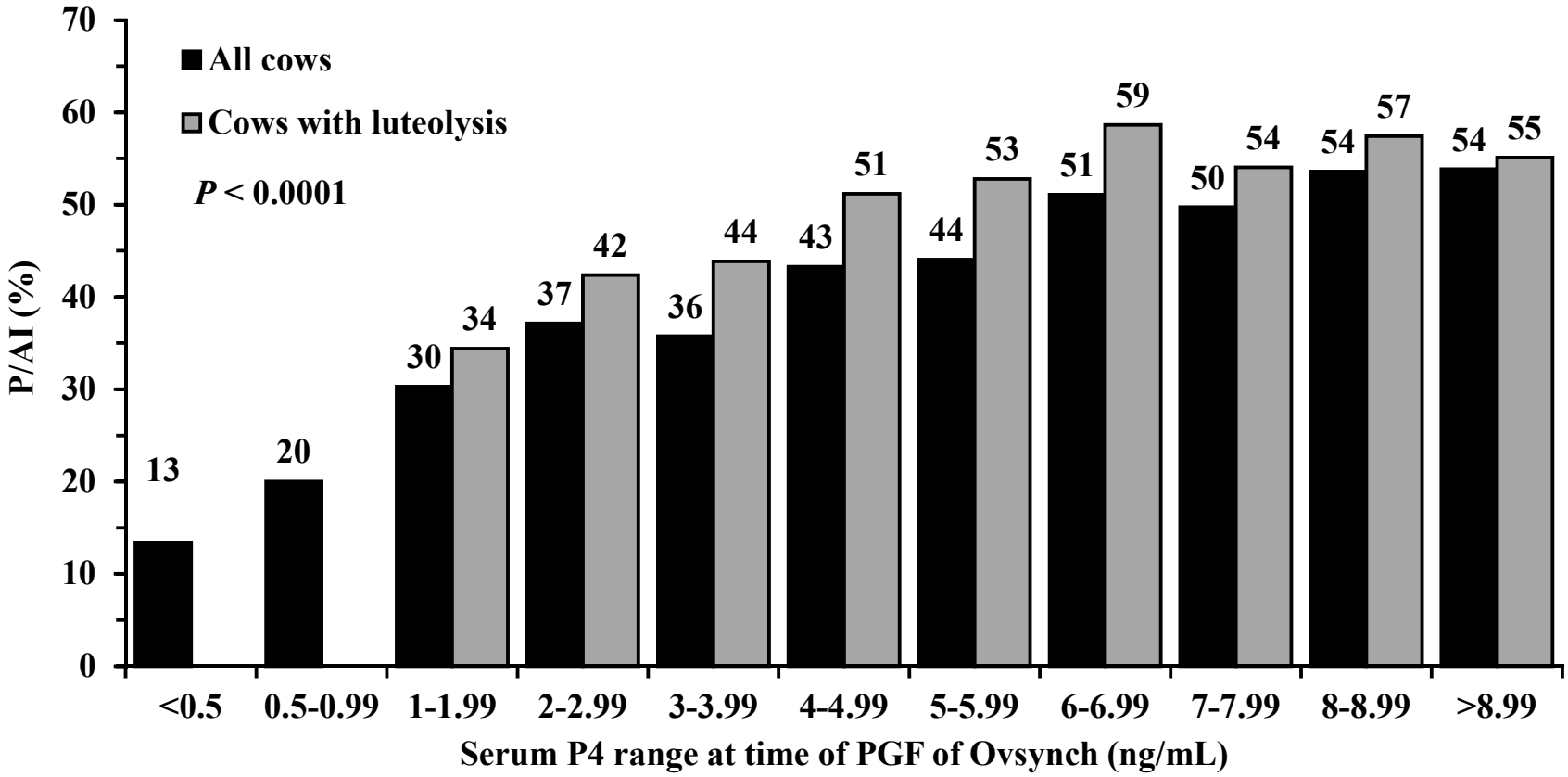

Figure 3.8. Relationship of progesterone concentrations at time of $PGF_{2\alpha}$ of Ovsynch and pregnancy / AI (%) in all cows (P<0.0001; n=2426) and in cows with complete luteolysis after the $PGF_{2\alpha}$ of Ovsynch (P<0.0001; n= 1670).

DISCUSSION

Fertility programs increase P/AI compared to AI following estrus [22,23,25]. Ovsynch [12], and other pre-synchronization programs [14,15,26] have differences in the logistics of inducing a new estrous cycle [9,14,15]. These differences directly affect the percent of cows that respond to the first GnRH of Ovsynch, and in turn affect P/AI outcomes. Pregnancy success after timed-AI is optimized when Ovsynch is initiated with the presence of a CL (in diestrus) [4-6]; [7-9], complete corpus luteum (CL) regression after the $PGF_{2\alpha}$ [10,11] and ovulation after final GnRH and following timed-AI [7,9,12]. To achieve this optimal scenario, days 6 or 7 were established as the best days of the estrous cycle to initiate Ovsynch [7,9,12]. Three pre-synchronization strategies were develop to increase percentage of cows on d 6 or 7 of the cycle: G6G [12], Double-Ovsynch [14] and Presynch-11 or -10 [15].

This study was designed to test two questions: (1) Does GnRH in a pre-synchronization program enhance P/AI? and (2) does a 5 d period from first GnRH of Ovsynch to $PGF_{2\alpha}$ allow for greater P/AI? This study used the most common pre-synchrony program for Ovsynch, Presynch-11, as control. Presynch-11 increased P/AI in several studies when compared to AI following estrus, and compared to Ovsynch alone [22,23]. Presynch-11 utilizes two injections of $PGF_{2\alpha}$ 14 d apart with the second $PGF_{2\alpha}$ administered 11 d prior to the start of Ovsynch (first GnRH). The disadvantages of this program are: (1) it only synchronizes the initiation of a new cycle if cows are cycling, and 2) there is significant variation in time to the new cycle after the second $PGF_{2\alpha}$ (2 to 6 d; ; [16,17]). Anovular cows do not seem to benefit from presynchronization protocols that only use $PGF_{2\alpha}$ -induced luteolysis since $PGF_{2\alpha}$ does not induce ovulation and formation of a new CL in cows without a CL [18]. We hypothesized that using a pre-synchronization strategy that include both $PGF_{2\alpha}$ and GnRH, would enhance P/AI in

lactating dairy cows. The second question from this study sought to determine if a 5 d period between the first GnRH and the $PGF_{2\alpha}$ of Ovsynch would enhance fertility. Our laboratory has unpublished data that a 5 d period increased circulating concentrations of E2 approximately 72 h after $PGF_{2\alpha}$ compared to the standard 7 d period. This also resulted in ovulation of a follicle with antral age, from initiation of a new follicular wave until ovulation, 2 d younger. So, in essence, the question deals with whether ovulating a younger, yet mature (high E2) follicle, results in greater fertility.

Effect of utilizing GnRH to pre-synchronize

The first $PGF_{2\alpha}$ of Presynch-11 increased the percent of cows with a functional CL at time of the second $PGF_{2\alpha}$ compared to the synchrony $PGF_{2\alpha}$ of G6G (Figure 6.2; 10 % point difference). This was likely due to an increased proportion of cows at a luteal stage of the estrous cycle compared to the initial $PGF_{2\alpha}$ of G6G, which was administered at a random stage of the estrous cycle. G6G utilizes $PGF_{2\alpha}$ and GnRH 2 d apart to initiate a new estrous cycle 6 d prior to Ovsynch [12]. Studies from our laboratory indicated that approximately 70 % of cows in a random stage of the cycle respond to both treatments [9,12].

GnRH administered prior to the pre-synchrony $PGF_{2\alpha}$ of Double Ovsynch increased the percentage of cows with a functional CL compared to Presynch-11 and G6G (Figure 6.2). The pre-synchrony GnRH of G6G increased the percent of cows with a functional CL at time of first GnRH of Ovsynch compared to Presynch-11 (Figure 6.2). The second $PGF_{2\alpha}$ of Presynch-11 did not increase the percentage of cows with a functional CL at the first GnRH of Ovsynch (Figure 6.2). Circulating progesterone in cycling cows at each critical point ($PGF_{2\alpha}$ of pre-synchrony, first GnRH and $PGF_{2\alpha}$ of Ovsynch) was measured (Figure 6.3). Circulating progesterone was

similar for each group on $PGF_{2\alpha}$ of pre-synchrony, greater for Presynch-11 at first GnRH likely due to a slightly more mature CL compared to other treatments. Progesterone concentrations were similar amongst groups at time of $PGF_{2\alpha}$ of Ovsynch with the exception of the DO5 group in which cows with accessory CL would be 2 d younger than the other groups. The ability of these programs to manipulate follicular development in order to enhance the opportunity to ovulate a DF at first GnRH of Ovsynch, was significantly greater than most data using Ovsynch alone [1,7,12]. Yet, DO7 had greater ovulation rates compared to G6G and Presynch-11 (Figure 6.4). Ovulation to the first GnRH of Ovsynch following G6G was 64 to 85 % [12,19,20], and it was greater compared to Ovsynch initiated in a random period of the estrous cycle [12]. Double Ovsynch adds another GnRH treatment 7 d prior to G6G, forming an entire Ovsynch protocol (GnRH – 7 d – $PGF_{2\alpha}$ – 2 d – GnRH) used to initiate a new estrous cycle 7 d prior the final timed-AI Ovsynch [14]. One of the benefits for using Ovsynch as pre-synchronization is the increase in the percentage of cows cycling prior to the timed-AI Ovsynch. Ovsynch is successful resuming cyclicity of anovular cows. Ovulation to first GnRH of Ovsynch following Double-Ovsynch pre-synchronization was 72 to 80 % [14,21].

Luteolysis at final $PGF_{2\alpha}$ of Ovsynch

Rates of luteolysis were similar between G6G and DO7. Presynch-11 had greater luteolysis rates compared to G6G. These luteolysis rates are greater than what most studies have reported with these programs with just one $PGF_{2\alpha}$ [10,27-30]. This study utilized two $PGF_{2\alpha}$ injections 8 h apart. In a previous study from our laboratory (J. R. Purlsey, unplub.), in which we utilized two $PGF_{2\alpha}$ injections 12 h apart when comparing 5, 6 or 7 d intervals between the first GnRH and $PGF_{2\alpha}$ of Ovsynch, lactating dairy cows had high rates of luteolysis in each interval;

however, this experiment had a small number of cows. In the current study, the DO5 group had approximately a 60% decrease in luteolysis rates compared to the other treatments. The only explanation for this dramatic decrease in luteolysis, even though two $PGF_{2\alpha}$ were utilized, is that in many cows, the d 5 accessory CL in this treatment was not sufficiently mature to respond to the double $PGF_{2\alpha}$ (Figure 6.6).

Pregnancies per AI

Despite the differences in the ability for these pre-synchrony programs to synchronize a new cycle, there were no differences in P/AI (Figure 6.7) in cows with luteolysis. In all cows, including cows that did not have luteolysis, P/AI was similar between Presynch-11, G6G and DO7, and predictably less in DO5 due to lack of luteolysis.

Relationship between progesterone at time of $PGF_{2\alpha}$ of Ovsynch and P/AI

There was a significant linear relationship between circulating progesterone at time of final $PGF_{2\alpha}$ of Ovsynch and P/AI (Figure 6.8). The greater the progesterone at time of final $PGF_{2\alpha}$ of Ovsynch the greater the chances for pregnancy. In high levels of progesterone, P/AI was greater than 55 % in cows with luteolysis. This indicates that lactating dairy cows can achieve very high fertility levels if progesterone is high during the growth of the ovulatory follicle.

CONCLUSIONS

Pregnancy/AI did not differ between GnRH-based pre-synchrony strategies compared to pre-synchrony strategy using only $PGF_{2\alpha}$. Moreover, adding GnRH 7 d prior to $PGF_{2\alpha}$ in G6G,

did not enhance P/AI in lactating dairy cows. Reducing 2 d between the first GnRH of Ovsynch and $PGF_{2\alpha}$ of Ovsynch (7 d vs. 5 d) decreased P/AI of lactating dairy cows treated with a modified Double Ovsynch program due to lack of luteolysis, even when using two $PGF_{2\alpha}$ treatments 8 h apart. Cows with a shorter interval between initiation of ovulatory follicle development and ovulation, that underwent complete luteolysis, still did not have greater fertility compared to the cows of the other treatments. There was a strong positive relationship between circulating concentrations of progesterone during the growth of the ovulatory follicle and P/AI. Presynch-11, G6G, and modified Double Ovsynch appeared to provide similar P/AI in all cows regardless of luteolysis status after $PGF_{2\alpha}$ of Ovsynch.

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CHAPTER 4

PRE-SYNCHRONIZATION OF LACTATING DAIRY COWS WITH $PGF_{2\alpha}$ AND GNRH SIMULTANEOUSLY, 7 DAYS PRIOR TO OVSYNCH HAVE SIMILAR OUTCOMES COMPARED TO G6G

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In Press

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ABSTRACT

The overarching objective of this study was to develop an alternative strategy for first and greater services that will improve fertility in lactating dairy cows for dairy operations limited by labor or other logistical constraints that make it difficult to use Presynch-11, G6G, or Double-Ovsynch. Our overall hypothesis was that simplification of a Presynch program through the combination of $PGF_{2\alpha}$ and GnRH on the same day (PG + G), 7 days before the first GnRH of Ovsynch, would allow for similar ovulation and luteolysis rate and pregnancies per AI (P/AI) compared with G6G. Lactating dairy cows 58 to 64 days in milk (first service; n = 114), and cows diagnosed not pregnant 39 days after previous AI (second + service; n = 122) were blocked by parity and service and randomly assigned to control or PG + G. Control cows received G6G (n = 116) that consisted of $PGF_{2\alpha}$, 2-day GnRH, 6-day GnRH, 7-day $PGF_{2\alpha}$, 56-hour GnRH, and 16-hour AI. Treated cows (PG + G; n = 121) received $PGF_{2\alpha}$ and GnRH, 7-day GnRH, 7-day $PGF_{2\alpha}$, 56-hour GnRH, and 16-hour AI. All cows received a second $PGF_{2\alpha}$ 24 hours after the $PGF_{2\alpha}$ of Ovsynch. First service cows received AI between 76 and 82 days in milk and second + service received AI 57 days after previous AI. Pregnancies/AI (n = 230) were similar in controls compared with treated cows on Day 35 (57 vs. 50%; P = 0.27) and Day 49 (54 vs. 47%; P = 0.33), respectively. Percent of cows ovulating after GnRH of the presynchronization was greater (P = 0.002) for controls vs. treated (80 vs. 58%); however, ovulation after first GnRH of Ovsynch was similar (67 vs. 68%; P = 0.86). Serum concentrations of progesterone were similar (P = 0.78) at the time of first GnRH of Ovsynch for control and treated cows (2.22 vs. 2.14 ng/mL). However, serum progesterone at the time of $PGF_{2\alpha}$ of Ovsynch was greater (P = 0.002) for control cows compared with treated cows (5.75 vs. 4.64 ng/mL). In summary, administering both $PGF_{2\alpha}$ and GnRH on the same day, 7 days before the start of Ovsynch,

appears to be a simple alternative that results in acceptable P/AI but potentially less progesterone during the growth of the ovulatory follicle.

INTRODUCTION

Fertility of lactating dairy cows treated with Ovsynch was enhanced when the first GnRH of the program induced ovulation [1]. This injection caused ovulation in approximately 60% of the lactating dairy cows when administered during random stages of the estrous cycle [1,2]. In comparison, ovulatory response was 80% or more when the first GnRH of Ovsynch was administered on Day 6 or 7 of the estrous cycle [1,2]. Administering GnRH on Day 6 or 7 of the estrous cycle compared with random stages resulted in greater percentages of cows with a new follicular wave, a new accessory CL, a functional CL at the $PGF_{2\alpha}$ of Ovsynch, and subsequent control of luteolysis [3]. In addition, initiating Ovsynch near Day 6 or 7 of the estrous cycle increases the chances for pregnancy after Ovsynch [1,2]. Three pre- synchronization strategies increased the chances for Ovsynch to be initiated near Day 6 or 7 of the estrous cycle (e.g., G6G [2,4,5], Double-Ovsynch [6–8], and Presynch-11 [9]) and increased pregnancy per artificial insemination (P/AI) compared with only Ovsynch or Presynch-14 or -12 and are now referred to as “fertility programs” [2,6–9].

Although fertility programs successfully improve reproductive performance, they are logistically challenging due the number of injections and the possibility for compliance problems. Farms that are restricted to administering injections on 2 days of the week due to labor constraints are limited to strategies that limit fertility outcomes (e.g., Ovsynch [10–12] and Presynch-14 or -12/ Ovsynch [13,14]). Creating a presynchronization strategy that can be administered on 2 days of the week with fertility outcomes greater that Ovsynch or Presynch-14/ Ovsynch would be advantageous for dairy producers.

Stevens et al. (1993) [15] compared the effect of the administering $PGF_{2\alpha}$ and GnRH simultaneously vs. $PGF_{2\alpha}$ with saline in lactating dairy cows on Day 8 or 10 of the estrous cycle to determine the effect of luteolysis. Administration of $PGF_{2\alpha}$ and GnRH simultaneously did not affect the luteolytic actions of $PGF_{2\alpha}$. Peters and Pursley (2003) [16] tested the effect of combining the final GnRH of Ovsynch with the $PGF_{2\alpha}$ of Ovsynch. There was no effect on luteolysis or ovulation rate compared with administering GnRH 36 hours after $PGF_{2\alpha}$. Yet, P/AI was decreased, and there was a trend for greater percentage of short cycles when the final GnRH was combined with the $PGF_{2\alpha}$ of Ovsynch.

The objective of this study was to develop a presynchronization strategy for Ovsynch that would limit treatments of GnRH and $PGF_{2\alpha}$ to 2 days per week, while producing fertility outcomes similar to that of G6G/Ovsynch. The hypothesis was that by combining $PGF_{2\alpha}$ and GnRH in a pre- synchronization strategy 1 week before Ovsynch, it would result in similar luteal and follicular outcomes after Ovsynch treatments, and P/AI compared with G6G/ Ovsynch.

MATERIALS AND METHODS

Cows, housing, feeding, and products

This experiment was conducted from May to August 2013 in a commercial dairy farm (Nobis Dairy Farm, St. Johns, MI, USA) that milked approximately 900 dairy cows 3 times daily. Herd milk production during this period averaged approximately 40 kg/cow/d. Cows were housed in free stall barns, fed a total mixed ration once daily, and had free access to feed and water. The total mixed ration consisted of corn, wheat and alfalfa silages, and corn-soybean meal-based concentrates formulated to meet or exceed nutrient recommendations for lactating dairy cows (NRC, 2001).

All treatments of $PGF_{2\alpha}$ (25-mg dinoprost tromethamine, Lutalyse, Zoetis) and GnRH (86-mg gonadorelin acetate, 2 mL of Fertagyl, Merck Animal Health) were administered with single dose syringes in semi-membranosus or semitendinosus muscles of cows by trained personnel from our laboratory. The Institutional Animal Care and Use Committee at Michigan State University approved all animal handling and procedures described in this article.

Experimental design

Healthy Holstein lactating dairy cows between 58 and 64 days in milk (DIM; first service; n = 114) and cows diagnosed not pregnant 39 days after previous AI (second and greater service; n = 122) were blocked by parity and service and randomly assigned to control (G6G [2]; n = 116) or treatment (PG + G; n = 121; see Figure 7.1). Controls were treated with $PGF_{2\alpha}$ followed in 2 days with GnRH, then 6 days later Ovsynch [10] (GnRH, 7-day $PGF_{2\alpha}$, 24-hour $PGF_{2\alpha}$, 32-hour -GnRH, and 16-hour AI) was initiated. Treatment cows received $PGF_{2\alpha}$ and GnRH in different sites seconds apart 7 days before the first GnRH of Ovsynch. All cows received timed-AI 16 hours after the final GnRH of Ovsynch at 76 to 82 DIM (first service) or 57 days after previous AI (second or greater services). Artificial insemination was performed by three AI technicians blind to treatments and using commercial semen from multiple sires purchased by the farm. Only one sire was used to inseminate weekly cohorts of cows; therefore, treatments were not confounded by an effect of service sire.

Ovarian ultrasonography, pregnancy diagnoses and blood samples

Ovarian structures (follicles and corpora lutea) were mapped and measured at time of the presynchrony GnRH and first GnRH of Ovsynch as previously described by Martins et al. [17]

using a MicroMaxx Sonosite ultrasound machine with a linear array transducer using 10-MHz frequency (Sonosite Inc., Bothell, WA, USA). Ovulation to each GnRH injection was determined by the disappearance of one dominant or codominant follicles (≥10 mm) previously visualized at the day of GnRH injection and the presence of a new CL 2 days later. Cows without a CL 2 days after the presynchrony GnRH but that had a CL at the time of first GnRH of Ovsynch were considered to have ovulated during the 5- day period before the first GnRH of Ovsynch.

Diagnoses of pregnancy were conducted by farm veterinarians blind to treatment performing transrectal ultrasonography with an Ibex Pro ultrasound machine with a 5 to 8-MHz linear array transducer (E.I. Medical Imaging, Loveland, CO, USA) on Day 35 and 49 after AI. Pregnancy was confirmed by embryo presence and heartbeat.

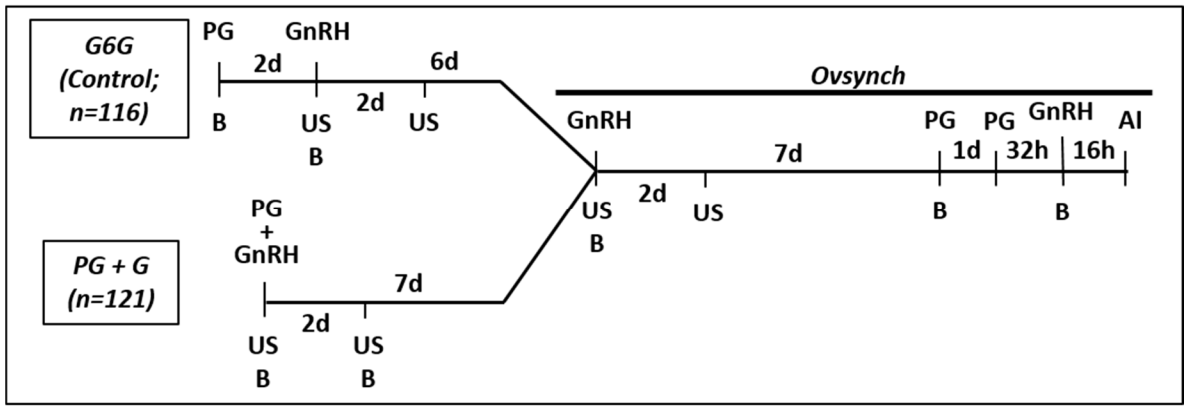

Figure 4.1: Schematic illustration of treatments, blood sample collections (B) and ultrasonography examinations of ovaries (US). PG= $PGF_{2\alpha}$.

Blood samples were collected by coccygeal venipuncture using Vacutainer tubes without anticoagulant (BD Vacutainer, Franklin Lakes, NJ, USA) on the day of pre-synchronization $PGF_{2\alpha}$ (n = 235) and 2 days later (n = 236) to determine cows' cycling status and luteolytic response for the presynchronization $PGF_{2\alpha}$ injection. Blood was also sampled at each injection of

Ovsynch (first GnRH, n = 234; $PGF_{2\alpha}$, n = 235; and final GnRH, n = 234). After collection, samples were refrigerated and transported to the laboratory and maintained at 4°C overnight. To assess circulating progesterone (P4) concentrations, serum was separated within 24 hours after collection by centrifugation at 2000 g for 20 minutes at 4°C and stored at -20°C for later hormonal analyses.

Progesterone assays and analysis of luteal function

Serum concentrations of P4 were quantified with RIA (Coat-a-Count, Siemens Diagnostic, Los Angeles, CA, USA). Intraassay and interassay CV were 7.7 and 6.5%, respectively. Cows with serum concentrations of P4 ≥ 1.00 ng/mL at the start of treatments were considered to have a functional CL. Cows with complete luteolysis were defined as cows with serum concentrations of P4 ≥ 1.00 ng/mL (with functional CL) at time of $PGF_{2\alpha}$ administration that decreased to < 0.50 ng/mL 2 days later. Cows with functional CL at the first GnRH of Ovsynch were defined as having at least one of the following: (1) ovulation to the presynchrony GnRH, (2) incomplete luteolysis after the presynchrony $PGF_{2\alpha}$ and serum P4 at time of first GnRH of Ovsynch ≥ 0.5 ng/mL, or (3) ovulation between the 5-day period before the first GnRH of Ovsynch and serum P4 at time of first GnRH of Ovsynch ≥ 0.5 ng/mL. Cows with functional CL at $PGF_{2\alpha}$ of Ovsynch were defined as having ovulation to at least one GnRH treatments before that, during the 5-day period before the first GnRH of Ovsynch, and no CL regression before $PGF_{2\alpha}$ of Ovsynch. Cows with complete luteolysis after $PGF_{2\alpha}$ of Ovsynch were characterized as cows with functional CL at time of $PGF_{2\alpha}$ administration that decreased to less than 0.50 ng/mL 2 days later.

Statistical analyses

Required total sample size used in the study was calculated using the z test for logistic regression of the G*Power software [18]. Power analysis indicated that a total of 217 subjects were needed to detect a 15%-point difference in P/AI between the two treatments using a two-tail test with $\alpha = 0.15$ and $\beta = 0.8$ in a binomial distribution.

Binomial variables were analyzed using logistic regression with a generalized linear mixed model implemented with the GLIMMIX procedure of SAS (version 9.4, SAS Inst., Inc., Cary, NC, USA). The model considered treatment (G6G or PG + G), parity (primiparous or multiparous), and service number (first or second and greater) as fixed effects and week as a random effect. Two-way interactions were only considered in the model if $P < 0.20$. There were $n = 6$ cows that left the herd between time of AI and first pregnancy diagnosis.

Continuous variables such as concentrations of P4 and ovulatory follicle size were analyzed using a linear mixed linear model applying the MIIXED procedure of SAS. The model used treatment (G6G or PG + G), parity (primiparous or multiparous) and service number (first or second and greater) as fixed effects and week as a random effect. Two-way interactions were only considered in the model if $P < 0.20$. Normal distribution of the residuals was tested with studentized residual plots for each variable. Residuals were considered normally distributed for all variables. All analysis used a two-tailed test and probabilities values were considered different when ≤ 0.05 and tendency for difference when ≤ 0.10.

Verification of the randomization of cows into treatments

Parity ($P = 0.81$) and service ($P = 0.66$) number were balanced between treatments. Mean ± standard error of the mean (SEM) parity number for all cows was 2.3 ± 0.1, ranging

from one to six. Service number mean ± SEM was 2.1 ± 0.1, ranging from one to seven. Days in milk at AI also did not differ (P = 0.99) between treatments. Overall mean ± SEM for DIM at AI was 131 ± 4, ranging from 76 to 351 DIM. Therefore, treatment effects were not confounded by parity, service number, and DIM at AI.

RESULTS

Effect of combining $PGF_{2\alpha}$ and GnRH on initiation of a new estrous cycle before Ovsynch

Administering $PGF_{2\alpha}$ concurrently with GnRH (PG + G) decreased the percentage of cows with complete luteolysis, ovulatory response to GnRH in the 2-day period after treatment, and percent of cows with a new estrous cycle compared with cows administered $PGF_{2\alpha}$ 2 days before GnRH (G6G; Table 7.1). A functional CL at time of treatment decreased ovulatory response to the GnRH in PG + G (Table 7.1). Ovulatory response was not different between G6G and PG + G for cows without a functional CL at time of treatment (Table 7.1).

Of cows that did not ovulate in the 2-day period after presynchronization treatments of GnRH, there were more (P = 0.03) cows in PG + G (17/50; 34%) compared with G6G (1/23; 4%) that ovulated in the 5 days period before the first GnRH of Ovsynch. All cows that ovulated in this period had luteolysis after presynchronization $PGF_{2\alpha}$ or no CL at that time. Approximately, one-half of these cows had double ovulations (8/18).

Table 4.1: Percentage of cows with functional corpus luteum (CL) at beginning of treatment, and effect of treatment (G6G vs. PG+G) on pre-synchronization outcomes.

	G6G (Control)	PG+G	P-value
Cows with functional CL at beginning of treatments[1], % (n/n)	56 (65/117)	66 (79/119)	0.11
Cows with complete luteolysis to the $PGF_{2\alpha}$ of the pre-synchronization[2], % (n/n)	89 (58/65)	71 (56/79)	0.01
Ovulation to the GnRH of the pre-synchronization, % (n/n)	80 (93/116)	58 (70/120)	0.002
Cows with functional CL at beginning of treatments[1], % (n/n)	95 (62/65)	54 (43/79)	<0.001
Cows without functional CL at beginning of treatments[1], % (n/n)	61 (31/51)	65 (26/40)	0.51
Size of the ovulatory follicle of the GnRH of the pre-synchronization[3], mm ± SEM	16.1 ± 0.4	16.2 ± 0.5	0.89
Double ovulation to the GnRH of the pre-synchronization (%)	28 (26/93)	16 (11/70)	0.14
Cows with new estrous cycle following pre-synchronization[4], % (n/n)	69 (80/116)	45 (54/120)	<0.001

[1]Cows with functional CL = Serum progesterone (P4) ≥ 1.00 ng/mL.
[2]Only cows with functional CL were used in this analysis. Complete luteolysis was consider when cows with functional CL at d of $PGF_{2\alpha}$ injection had serum P4 < 0.5 ng/mL 2 d later.
[3]Only cows with single ovulations used on calculations.
[4]Cows with a new estrous cycle = (1) cows with a functional CL at beginning of treatment that had complete luteal regression and ovulation for the $PGF_{2\alpha}$ and GnRH pre-synchronization injections, respectively, or (2) cows without functional CL at beginning of treatment that ovulated after the GnRH injection of the pre-synchronization

Effect of treatment on ovarian function at, and following, first GnRH of Ovsynch

Administering $PGF_{2\alpha}$ simultaneously with GnRH decreased (P = 0.03) the percentage of cows with functional CL at time of first GnRH of Ovsynch (Table 7.2). Serum concentrations of P4 were not different between treatments (Table 7.2 and Figure 7.2). Ovulatory follicle diameter was greater (P = 0.002) for PG + G compared with G6G in cows with single dominant follicles that ovulated after the first GnRH of Ovsynch (Table 7.2). There were no treatment differences in ovulatory response after first GnRH of Ovsynch (Table 7.2). Cows that responded to the

presynchronization treatments and were on Day 6 or 7 of the cycle (n = 134) at first GnRH of Ovsynch had greater ovulatory response (81% vs. 51%; P = 0.001) and smaller ovulatory follicles (14.6 ± 0.2 vs. 15.6 ± 0.3 mm; P = 0.008) compared with cows that did not have luteolysis and/or ovulation following presynchronization treatment (n = 102). Cows on Day 6 of the estrous cycle (G6G) ovulated smaller follicles (13.8 ± 0.3 vs. 15.5 ± 0.3 mm; P < 0.001) and had lower serum P4 compared (1.76 ± 0.08 vs. 2.37 ± 0.14 ng/mL; P < 0.001) with cows on Day 7 (PG + G) of the estrous cycle.

Table 4.2: Effect of treatment (G6G vs. PG+G) on luteal and follicular functions at first GnRH of Ovsynch and response to first GnRH of Ovsynch.

	G6G (Control)	**PG+G**	**P-values**
Cows with functional CL at the first GnRH of the Ovsynch [1], % (n/n)	91 (104/115)	76 (90/119)	0.03
Serum P4 at the 1st GnRH of the Ovsynch, ng/mL ± SEM	2.22 ± 0.18	2.14 ± 0.18	0.78
Ovulation to the 1st GnRH of Ovsynch, % (n/n)	67 (78/116)	68 (82/120)	0.86
Size of the ovulatory follicle of the 1st GnRH of the Ovsynch[2], mm ± SEM	14.3 ± 0.3	15.7 ± 0.3	0.002
Double ovulation to the 1st GnRH of Ovsynch, % (n/n)	22 (17/78)	23 (19/82)	0.79

[1]Cows with functional corpus luteum (CL) at the 1st GnRH of Ovsynch were defined as having at least one of the following: (1) ovulation to the pre-synchrony GnRH, (2) no complete luteolysis after the pre-synchrony $PGF_{2\alpha}$ and serum progesterone (P4) at time of 1st GnRH of Ovsynch ≥ 0.5 ng/mL, or (3) ovulation between the 5 d period before the 1st GnRH of Ovsynch and serum P4 at time of 1st GnRH of Ovsynch ≥ 0.5 ng/mL.
[2]Only cows with single ovulation.

Effect of treatment on luteal function at time of $PGF_{2\alpha}$ of Ovsynch

Percent of cows with functional CL at time of $PGF_{2\alpha}$ of Ovsynch did not differ between treatments (Table 7.3). Mean serum concentrations of P4 at time of $PGF_{2\alpha}$ of Ovsynch was greater (P = 0.002) for cows treated with G6G than cows treated with PG + G (Table 7.3) due to

treatment differences in first service cows (Figure 7.2). When treatments were combined, primiparous cows had greater (P = 0.003) serum P4 at $PGF_{2\alpha}$ of Ovsynch compared with multiparous cows (5.87 ± 0.33 vs. 4.78 ± 0.22 ng/mL; respectively).

Table 4.3: Effect of treatment (G6G vs. PG+G) on luteal function at $PGF_{2\alpha}$ of Ovsynch and response to $PGF_{2\alpha}$ of Ovsynch.

	G6G (Control)	PG+G	P-values
Cows with functional CL at the $PGF_{2\alpha}$ of Ovsynch [1], % (n/n)	94 (108/115)	93 (111/120)	0.67
Serum P4 at the $PGF_{2\alpha}$ of Ovsynch, ng/mL ± SEM	5.75 ± 0.28	4.64 ± 0.24	0.002
Cows with complete luteolysis after the $PGF_{2\alpha}$ of Ovsynch[2], % (n/n)	96 (103/107)	97 (108/111)	0.68

[1]Cows with functional corpus luteum (CL) at $PGF_{2\alpha}$ of Ovsynch were defined as having ovulation to at least one GnRH treatment or during the 5 d period before the 1st GnRH of Ovsynch and no CL regression prior to $PGF_{2\alpha}$ of Ovsynch.
[2]Only cows with functional CL were used in this analysis. Complete luteolysis was consider when cows with functional CL at d of $PGF_{2\alpha}$ injection had serum progesterone (P4) < 0.5 ng/mL 2 d later.

Effect of treatment on luteolysis to $PGF_{2\alpha}$ of Ovsynch

There was no effect of treatment on percentage of cows with complete luteolysis (Table 7.3). Only seven cows with functional CL at time of $PGF_{2\alpha}$ of Ovsynch did not have complete luteolysis. Six of seven cows had serum P4 between 0.50 and 1.00 ng/mL. A greater (P = 0.02) percent of multiparous cows had complete luteolysis compared with primiparous cows (99% vs. 93%; respectively).

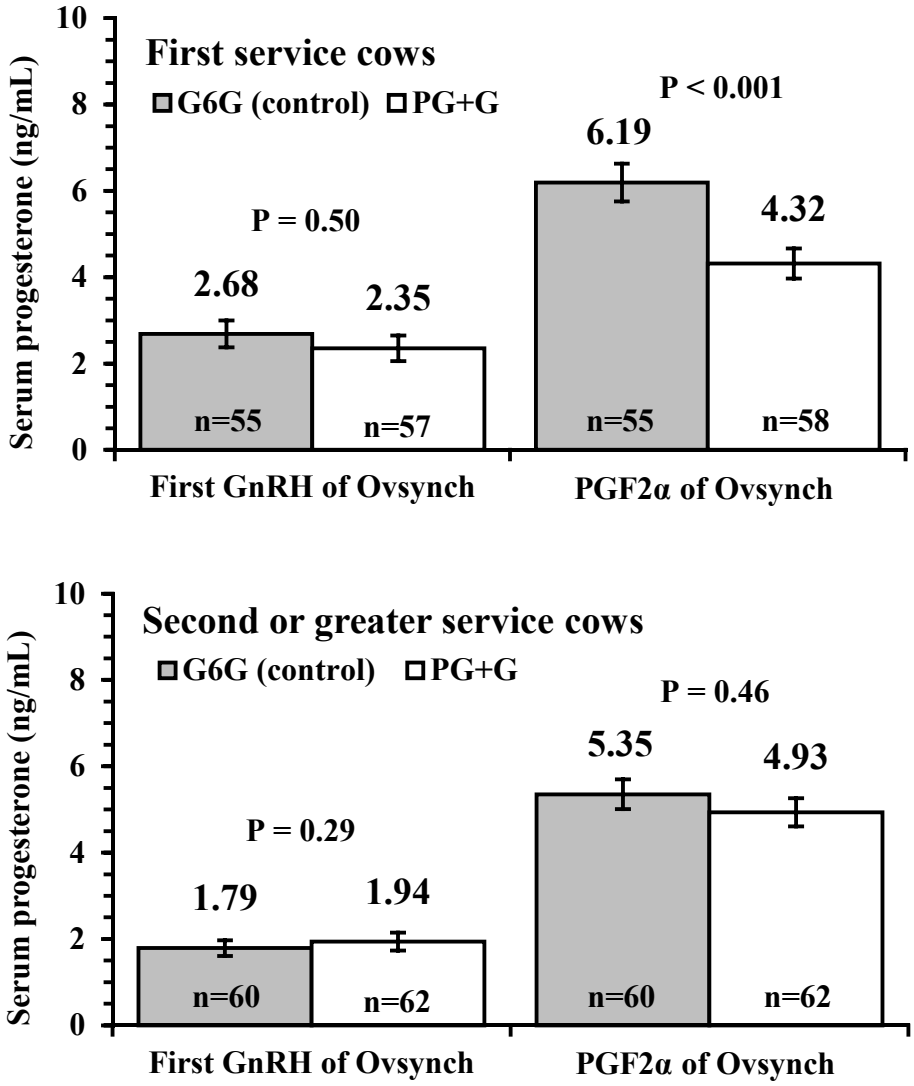

Figure 4.2: Effect of treatment (G6G vs. PG+G) on serum concentrations of progesterone (ng/mL) at first GnRH of Ovsynch and at $PGF_{2\alpha}$ of Ovsynch for first and second or greater service cows. Interaction treatment and service at first GnRH of Ovsynch: P = 0.22 and at $PGF_{2\alpha}$ of Ovsynch: P = 0.03.

Effect of treatment on pregnancies per AI at 35 and 49 days after AI

Treatment did not affect P/AI on 35 or 49 days after AI (Figure 7.3). There was no effect of service number on P/AI at 35 (P = 0.67; 51% vs. 55% for first vs. greater services, respectively) or 49 days after AI (P = 0.53; 49% vs. 52% for first vs. greater services, respectively). There was no effect of parity (P = 0.17) on P/AI at 35 days after AI (60% vs. 50%

for primiparous vs. multiparous, respectively). However, primiparous cows had a trend (P = 0.06) for greater P/AI at 49 days after AI compared with multiparous cows (59% vs. 46%, respectively). Percentage of cows with pregnancy losses between 35 and 49 days after AI was 4% and was not affected by treatment (P = 0.69), parity (P = 0.27), or service (P = 0.45).

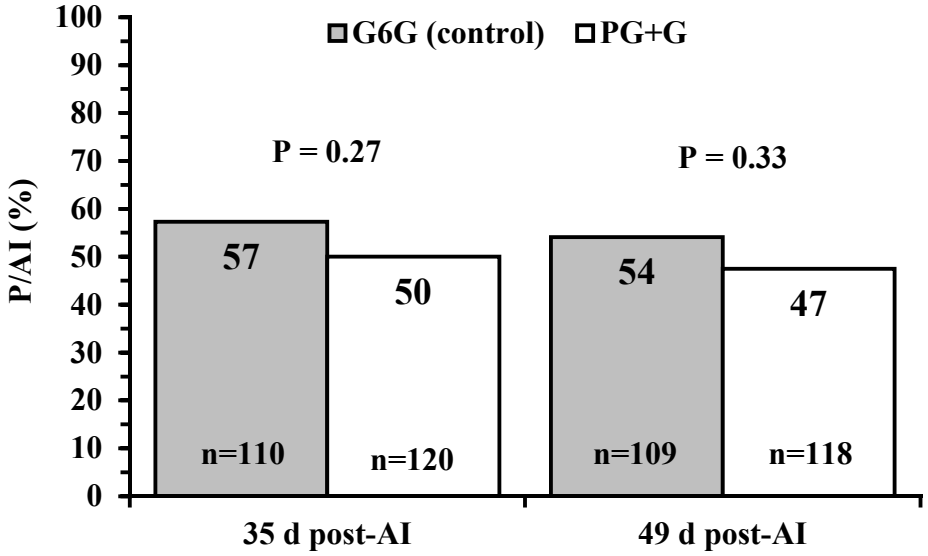

Figure 4.3: Effect of treatments (G6G vs. PG+G) on pregnancies per AI (P/AI) at 35 and 49 d post-AI. Interaction service by treatment was not significant for 35 d post-AI (P = 0.82) and for 49 d post-AI (P = 0.76).

DISCUSSION

The objective of this study was to develop a presynchronization strategy for Ovsynch that limits treatments of GnRH and $PGF_{2\alpha}$ to 2 days per week and results in fertility outcomes similar to that of G6G/Ovsynch. G6G is administered on four different days of the week. Presynch-10/11 requires 3 days of the week to administer. Pregnancies per AI were not different between treatments but numbers of cows per treatment was limited resulting in approximately a 35% probability of a type-II error. Pregnancies per AI on Day 35 and 49 after AI for G6G were greater (57% and 54%) than in previous studies that used G6G (35%–50%) [2,4,5]. The high-

luteolysis rate after the final $PGF_{2\alpha}$ of Ovsynch likely influenced P/AI in this experiment. Cows without complete luteolysis after $PGF_{2\alpha}$ of Ovsynch have reduced chances of pregnancy [19]. The use of two $PGF_{2\alpha}$ injections 1 day apart at final $PGF_{2\alpha}$ was very effective in inducing complete luteolysis in cows treated with PG + G and G6G and is in agreement with previous studies [20]. In spite of the high P/AI of G6G, cows treated with PG + G had similar P/AI to G6G treatment, indicating that PG + G could be a simpler alternative for timed-AI programs for first or greater services for farms that want to use the same weekdays of animal handling that are used for the Ovsynch protocol.

We hypothesized that combining $PGF_{2\alpha}$ and GnRH in a presynchronization strategy 1 week before Ovsynch would result in similar luteal and follicular outcomes after Ovsynch treatments compared with G6G/Ovsynch. Results indicated that ovulation to the first GnRH of Ovsynch were not different between G6G and PG + G. This partially supported our main hypothesis. The probability of a type-II error was approximately 24% because values are in a part of the curve with more power. Similar ovulation rates to first GnRH of Ovsynch may help to explain similar percentages of cows pregnant for G6G and PG + G. Ovulation to the first GnRH of Ovsynch is a major factor for the success of timed-AI programs [1]. Previous studies reported that cows that ovulated to the first GnRH of Ovsynch had greater fertility after timed-AI compared with cows that did not ovulate after this injection [2,21,22]. G6G [2] and other fertility programs (Presynch-10 or 11 and Double-Ovsynch) [6,9,23] were developed to increase percentage of cows on Day 6 or 7 of the estrous cycle to optimize ovulatory response to the first GnRH of Ovsynch, increase circulating concentrations of P4 during the development of the ovulatory follicle, and improve pregnancies per timed-AI. Although PG + G had similar ovulation rate compared with G6G, percentage of cows on Day 6 or 7 at the first GnRH of

Ovsynch was reduced for PG + G due to a decrease in the percentage of cows that started a new cycle after PG + G.

The combination of $PGF_{2\alpha}$ and GnRH reduced the effect of both injections responses after PG + G presynchronization program. The underlying mechanism that reduced the lutelolysis rate after the $PGF_{2\alpha}$ of the presynchronization for cows treated with PG + G is not clear. In both treatments, $PGF_{2\alpha}$ was administered on a random day of the estrous cycle. Only cows with functional CL were used to analyze percentage of cows with complete luteolysis. Serum P4 at time of $PGF_{2\alpha}$ was not different between cows with complete luteolysis vs. without luteolysis, and it probably did not interfere in the effectiveness of $PGF_{2\alpha}$ in contrast with a previous experiment that had lower probability of complete luteolysis for cows with lower P4 at time of $PGF_{2\alpha}$ [19]. Interestingly, we could not find published data to support the possibility that the LH surge induced by the GnRH administration disrupted or delayed the luteolysis process in the PG + G treatment. In previous data [15], simultaneous administration of GnRH did not affect $PGF_{2\alpha}$ -induced luteolysis in 16 cows on Day 8 or 10 of the cycle, and all cows (16/16) reached P4 < 0.5 ng/mL in 1 day post $PGF_{2\alpha}$. In this study, only 70% (56/79) of cows treated with PG + G reached P4 levels < 0.5 ng/mL 2 days after the injections, and only 2/23 had P4 levels between 0.50 and 1.00 ng/mL 2 days after $PGF_{2\alpha}$. Cows treated with G6G in our experiment appeared to have a greater luteolysis rate (89%) to what was previously reported on the first article of G6G (77%, [2]) and in the last $PGF_{2\alpha}$ of Ovsynch (77%, [19]). Therefore, additional studies with larger number of experimental units are necessary to determine whether GnRH in combination with $PGF_{2\alpha}$ reduces luteolysis rate or whether a type-II error influenced our results.

The addition of 2 days between the administration of GnRH and $PGF_{2\alpha}$ of G6G treatment also improved the ovulatory response of GnRH when compared with PG + G. This result may be

attributed to the reduction of serum P4 for cows with functional CL. During luteolysis, serum P4 decrease induces an increase of LH pulse frequency [24], which allows the continuous development of a dominant follicle (DF) and avoids a DF turnover. Cows with functional CL treated with G6G had a greater chance to have a DF at time of GnRH of the presynchronization compared with PG + G. The lower ovulatory response to the GnRH of PG + G for cows with functional CL may be attributed to the reduction of the magnitude of the LH surge. Giordano and collaborators [25,26] reported that high-serum P4 significantly suppressed GnRH-induced LH surge and reduced the magnitude of the LH surge.

The presence of a functional CL might also have negatively affected the ovulatory response for the first GnRH of Ovsynch for cows treated with G6G because they had a greater percentage of functional CL at the first GnRH of Ovsynch compared with PG + G. Previous studies already reported that the presence of a functional CL at the time of GnRH significantly reduced ovulatory response for first GnRH of Ovsynch [26-28]. In the present study, the ovulation rate to the first GnRH to Ovsynch for cows treated with G6G appeared to be similar to what was previously reported for other studies that used G6G (64% [29] and 69% [5]), Double-Ovsynch (72%, [6]), Presynch-11 (61% [9]), and Presynch-10 (70% [30]). However, it was lower than our previous studies using G6G in first service cows (85%, [2,31]). In agreement with previous studies [1,2], cows on Day 6 or 7 of the estrous cycle had greater ovulation rate to the first GnRH of Ovsynch compared with cows in other times of the estrous cycle.

Because the percentage of cows with functional CL was reduced in cows treated with PG + G, we were expecting a lower serum P4 at time of first GnRH of Ovsynch for cows treated with PG + G compared with G6G; however, treatments had similar serum P4. This result appeared to be caused by a smaller percentage of cows with complete luteolysis after PG + G

treatment, which might have resulted in a greater proportion of cows in mid-estrous cycle at time of first GnRH of Ovsynch. Cows in mid-estrous cycle at first GnRH of Ovsynch had lower percentage of cows with functional CL and serum P4 at time of $PGF_{2\alpha}$ of Ovsynch and lower fertility after timed-AI compared with cows between Day 5 and 9 of the cycle [32]. In the present study, it appears that this greater percentage of cows in mid-estrous cycle might not have influenced the overall outcome of the PG + G program since percentage of cows with functional CL at time of $PGF_{2\alpha}$ of Ovsynch and P/AI after timed-AI was similar between the treatments.

PG + G might be a good substitution for Presynch-14 or -12 [13,14]. These programs use the same weekdays for injections as Ovsynch but take longer to execute. In addition, these protocols are commonly used in combination with AI after estrus detection after the $PGF_{2\alpha}$ administration of the presynchronization and subsequent timed-AI for cows that were not detected in estrus [33-36]. Fertility for cows inseminated following estrus after $PGF_{2\alpha}$ of Presynch has been reported to be similar [33-35] or reduced [36] compared with cows that only received timed-AI. On the other hand, the use of the PG + G program for first and subsequent inseminations with the addition to detection of estrus between PG + G presynchrony and first GnRH of Ovsynch may be a good strategy for farms that want to limit the number of days in the week they are handling cows. PG + G increased the percent of cows that ovulated during the 5 days before the first GnRH of Ovsynch. These cows would likely be in early stages of follicle development (predeviation) at time of PG + G and may have normal fertility after AI after estrus detection [37].

CONCLUSIONS

Results from the present experiment indicate that administering both $PGF_{2\alpha}$ and GnRH on the same day, 7 days before the start of Ovsynch (PG + G) may offer a reasonable alternative to

more complex fertility programs to enhance P/AI for first and subsequent services. The protocol PG + G appeared to provide sufficient ovulatory response to the first GnRH of Ovsynch and concentrations of progesterone at the time of $PGF_{2\alpha}$ of Ovsynch. The increase in the percent of cows in the PG + G treatment that ovulated during the 5 days before the first GnRH of Ovsynch is a concern, and need to be further investigated.

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CHAPTER 5

THE EFFECT OF PRE-SYNCHRONIZATION WITH $PGF_{2\alpha}$ AND GNRH SIMULTANEOUSLY, 7 D PRIOR TO OVSYNCH, COMPARED TO PRESYNCH-10/OVSYNCH ON LUTEAL FUNCTION AND FIRST SERVICE PREGNANCIES PER AI

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ABSTRACT

Pregnancy per artificial insemination (P/AI) following Ovsynch is optimized when cows ovulate to the first GnRH of Ovsynch. Therefore, fertility programs intend to pre-synchronize cows to d 6 or 7 of the estrous cycle to increase the chances of ovulation of a first wave dominant follicle to the first GnRH of Ovsynch. The hypothesis of this experiment states that simplification of a pre-synchronization program through the combination of $PGF_{2\alpha}$ and GnRH on the same day and 7 d before Ovsynch, would allow for similar P/AI compared to Presynch-10. Lactating dairy cows (n = 432) 41 to 47 DIM were assigned in a random fashion to two treatments within parities for first service. Control cows received Presynch-10/Ovsynch consisting of the following: $PGF_{2\alpha}$ −14 d− $PGF_{2\alpha}$ −10 d− GnRH −7 d− $PGF_{2\alpha}$ −56 h– GnRH −16 h− AI. Treated cows received $PGF_{2\alpha}$ and GnRH –7 d– GnRH −7 d− $PGF_{2\alpha}$ −56 h− GnRH –16 h− AI. All cows received a supplemental injection of $PGF_{2\alpha}$ 24 h after the $PGF_{2\alpha}$ of Ovsynch to enhance complete luteolysis. All cows received timed-AI between 75 and 81 DIM. Blood was collected to assess circulating concentrations of progesterone (P4), and number and size of corpora lutea (CL) were recorded using ultrasonography on d of $PGF_{2\alpha}$ of Ovsynch. PG+G had similar P/AI at 28 (46 vs. 48%), 35 (43 vs. 43%), 49 (39 vs. 39%) and 77 d post-AI (38 vs. 39%) compared to Presynch-10. There were no differences in P/AI in primiparous vs. multiparous at 28 (52 vs. 45%), 35 (48 vs. 41%), 49 (45 vs. 37%) and 77 d post-AI (43 vs. 36%). There was no difference in treatments in percentage of cows with functional CL at $PGF_{2\alpha}$ of Ovsynch, total luteal area (mm^2), or serum concentrations of P4 at time of $PGF_{2\alpha}$ of Ovsynch, regardless of parity. Number of CL had a tendency to be greater for multiparous PG+G vs. Presynch-10 cows (2.34 ± 0.09 vs. 2.15 ± 0.08) but not in primiparous cows (1.95 ± 0.10 vs. 1.98 ± 0.11). In summary, administering both $PGF_{2\alpha}$ and GnRH on the same d, 7 d before the start of Ovsynch, appears to be a simple and effective

alternative to Presynch-10 Ovsynch.

INTRODUCTION

Successful synchronization programs ensure luteolysis and subsequent ovulation of a mature dominant follicle in precise period to allow for a successful timed-AI. Progress has been made in the past decade in improving fertility of lactating dairy cows through manipulating ovarian development prior to AI [1]. The improvement in fertility is considered to be due to greater control of the hormone milieu, primarily progesterone (**P4**), and antral age of the ovulatory follicle [2], which is regulated mainly by ovulation to the first GnRH of Ovsynch [3] and the development of an accessory corpus luteum (**CL**). Cows that ovulated to this injection had greater fertility after timed-AI compared to cows that did not ovulate [4-6].

Initiating Ovsynch on d 6 or 7 of the estrous cycle maximizes ovulatory response following the first GnRH of Ovsynch, controls follicle and CL development, and increases pregnancy outcome following timed-AI [2]. Three pre-synchronization strategies that increase the percent of cows on d 6 or 7 of the estrous cycle at the first GnRH of Ovsynch are: G6G [4,7,8], Double-Ovsynch [9-11] and Presynch-10 or 11 [12]. Presynch10, for example, is designed to synchronize cows to d 6 or 7 of the cycle, since most cycling cows will be in estrus 3 to 4 d following the second $PGF_{2\alpha}$ if they respond to the second injection of $PGF_{2\alpha}$. These programs successfully improve reproductive performance of lactating dairy cows following first service compared to cows inseminated following estrus [13,14], and are now referred to as "fertility programs for lactating dairy cows." Yet, fertility programs can be logistically challenging due the number of injections, and the possibility for compliance problems. Dairy operations that are limited to administering pre-synchronization injections on the same day of the week as Ovsynch are restricted to use only Presynch-14 or -12 [15,16]. These programs limit fertility success

following timed-AI because they pre-synchronize cows to d 8 to 10 of the estrous cycle, when first wave dominant follicles begin to become atretic, progesterone is increasing, and chances of ovulation to the first GnRH of Ovsynch begins to be reduced compared to d 6 or 7. Thus fewer cows are likely to induce a new accessory CL and initiate the timing of a new follicular wave in response to a GnRH-induced LH surge during this period.

Creating a simpler pre-synchronization program for first service with greater fertility outcomes similar to G6G, Double Ovsynch and Presynch-10/11 may be beneficial for dairy producers. It is not clear if administration of $PGF_{2\alpha}$ simultaneously with GnRH 7 d prior to Ovsynch (PG+G) would result in the induction of a new cycle and allow for cows to be on d 7 of the estrous cycle at time of the first GnRH of Ovsynch. The administration of $PGF_{2\alpha}$ and GnRH concurrently in cows on d 8 or 10 of the estrous cycle did not affected luteolytic actions of $PGF_{2\alpha}$ when compared to $PGF_{2\alpha}$ and saline [17]. Thus, the present study aimed to determine differences on pregnancy/AI (**P/AI**) and serum concentrations of P4 at time of the $PGF_{2\alpha}$ of Ovsynch between PG+G and Presynch-10 in first-service lactating dairy cows. Presynch-10 was chosen as the controls in this study since synchronized cows have the opportunity to be near the same d of the cycle at the start of Ovsynch as cows synchronized to PG+G. We hypothesized that pre-synchronization with PG+G would have similar P/AI compared to Presynch-10 in first-service lactating dairy cows.

MATERIALS AND METHODS

This experiment was conducted from May of 2012 and January of 2013 on a commercial dairy farm (Nobis Dairy Farm, St. Johns, MI) with approximately 900 lactating dairy cows that were milked 3 times daily. Average herd milk production during this period was approximately

40 kg/cow/d. Cows were housed in free stall barns, fed a TMR once daily, and had free access to feed and water. The TMR consisted of corn, wheat and alfalfa silages and corn-soybean meal-based concentrates formulated to meet or exceed nutrient recommendations for lactating dairy cows (NRC, 2001). Trained personnel from our laboratory administered all injections of $PGF_{2\alpha}$ (0.5 mg cloprostenol, 2 mL of Estrumate, Merck Animal Health) and GnRH (86 μg gonadorelin, 2 mL of Fertagyl, Merck Animal Health) with single dose syringes in semimembranosus or semitendinosus muscles of cows. The Institutional Animal Care and Use Committee at Michigan State University approved all procedures described in this manuscript.

Experimental design

Weekly cohorts of healthy lactating dairy cows (n = 432) between 41 and 47 DIM were blocked by parity and randomly assigned to two treatments for first service. Control cows (n = 214) received Presynch-10 consisting of 2 injections of $PGF_{2\alpha}$ 14 d apart with the second injection 10 d prior to the first GnRH of Ovsynch (GnRH – 7 d – $PGF_{2\alpha}$ – 24 h – $PGF_{2\alpha}$ – 32 h – GnRH – 16 h – AI; Figure 8.1). Treated cows (PG+G; n = 218) received $PGF_{2\alpha}$ and GnRH in different sites seconds apart 7 d prior to the first GnRH of Ovsynch. All cows received timed-AI 16 h after the final GnRH of Ovsynch at 75 to 81 DIM. Three AI technicians blind to treatments performed artificial insemination. Commercial semen from multiple sires purchased by the farm was utilized. Only one sire was used to inseminate weekly cohorts of cows; therefore, treatments were not confounded by an effect of service sire. A second $PGF_{2\alpha}$ was administered 24 h following the first $PGF_{2\alpha}$ of Ovsynch to ensure luteolysis. Timing of final GnRH of Ovsynch remained at 56 h following the first $PGF_{2\alpha}$.

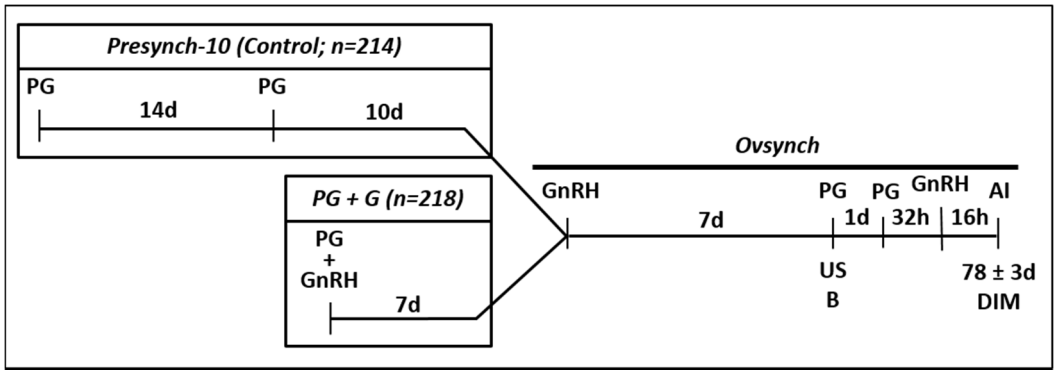

Figure 5.1. Schematic illustration of experimental design with time of hormone injections ($PGF_{2\alpha}$ and GnRH), blood collection (B) and ultrasonography examination of ovaries (US) for each treatment, Presynch-10 (control) vs. PG+G. PG= $PGF_{2\alpha}$; AI = artificial insemination.

Ovarian ultrasonography and pregnancy diagnoses

Ovaries were mapped in a random subset of cows on d of first $PGF_{2\alpha}$ of Ovsynch (n = 374; Figure 1) by transrectal ultrasonography as previously described by Martins et al. [18] using a Sonosite MicroMaxx ultrasound with a 5-10 MHz multi-frequency linear array probe (Sonosite, Bothell, WA). Height (**H**) and width (**W**) of the largest size of each follicle with average diameter > 7 mm and CL with average diameter > 12 were measured with build-in calipers. Fluid-filled cavities in CL were measured in the same fashion. The area of each CL and each cavity (if present) was calculated with the following equation: $0.5\ H \times 0.5\ W \times \pi$. The luteal area of each CL was calculated subtracting the cavity area from the total CL area. Total luteal area was the sum of the luteal area of all CL. Diameter of each follicle was calculated by the average of H and W of each follicle. Follicles ≥ 25 mm were considered cysts based on previous data [19-21] and were not considered to the analysis of largest follicle size.

Trained personnel from our laboratory blind to treatment performed pregnancy diagnoses on d 28 post-AI using the SonoSite MicroMaxx ultrasound described above. Farm veterinarians blind to treatment performed pregnancy diagnoses on d 35, 49 and 77 after AI using an Ibex Pro

ultrasound machine (E.I. Medical Imaging, Loveland, CO). Pregnancy was confirmed by the heartbeat of an embryo.

Blood collection, hormone assays and analysis of luteal function

Blood samples were collected from the coccygeal vein or artery of a subset of cows at time of first $PGF_{2\alpha}$ of Ovsynch (n = 404) and 28 d post-AI (n = 327) using Vacutainer tubes without anticoagulant (BD Vacutainer, Franklin Lakes, NJ) to measure serum concentrations of P4. Following collection, samples were refrigerated, transported to our laboratory and maintained at 4 °C overnight. Serum was separated within 24 h after collection by centrifugation at 2000 x g for 20 min at 4 °C and stored at -20 °C for later P4 analyses.

Serum concentrations of P4 were quantified with RIA (Coat-a-Count, Siemens Diagnostic, Los Angeles, CA). Intra- and inter-assay CV were 9.1 and 6.0 %, respectively. Cows with serum concentrations of P4 ≥ 1.00 ng/mL were considered to have a functional CL.

Statistical analyses

Total number of cows necessary in the study was calculated by power analysis using the z test for logistic regression of G*Power [22]. A total of 432 subjects were needed to detect a 10 % difference in P/AI between treatments (45 vs. 55%) using a two-tail test with $\alpha = 0.05$ and $\beta = 0.80$ in a binomial distribution.

Binomial variables were analyzed using logistic regression with a generalized linear mixed model implemented with the GLIMMIX procedure of SAS (Version 9.4, SAS Inst., Inc., Cary, NC). The model considered treatment (Presynch-10 or PG+G) and parity category (primiparous or multiparous) as fixed effects and week as a random effect. Two-way interaction of treatment

and parity category was only considered in the model if $P < 0.20$. Continuous variable were analyzed using a linear mixed linear model applying the MIIXED procedure of SAS with the same fixed effects and random effect as describe for the GLIMMIX procedure. Treatment and parity number interaction was only considered in the model if $P < 0.20$. Normal distribution of the residuals was tested with studentized residual plots for each variable. Residuals were considered normally distributed for all variables. Distribution of cows across different ranges of serum P4 and follicle diameter were analyzed by chi-square test using the FREQ procedure of SAS. Effects of total luteal area and serum concentrations of P4 at time of $PGF_{2\alpha}$ of Ovsynch were tested using regression analyses with the REG procedure of SAS. All analysis used a two-tailed test and probabilities values were considered different when $P \leq 0.05$.

RESULTS

Percent of cows with functional CL, total luteal area and concentrations of serum P4 at time of first $PGF_{2\alpha}$ of Ovsynch did not differ between treatments (Table 8.1). No interaction of treatment and parity was observed in luteal measurements ($P = 0.51$). Figure 8.2 describes the differences in distribution of cows (%) within treatment across ranges of P4 at first $PGF_{2\alpha}$ of Ovsynch. There was a shift in percentage of cows in the medium low (3 to 4.99 ng/mL) and high P4 ranges (≥ 7 ng/mL) between treatments. There was a relationship between total luteal area and serum concentration of P4 at first $PGF_{2\alpha}$ (Figure 8.3). There was no effect of treatment on the percent of cows with 1, 2, or 3+ CL at that time; however, there were strong relationships between CL number, luteal volume and size of the largest follicle at first $PGF_{2\alpha}$ of Ovsynch (Table 8.2).

Table 5.1: Effect of treatment, Presynch-10 (control) vs. PG+G, in a subset of first service lactating dairy cows with functional CL at time of $PGF_{2\alpha}$ of Ovsynch on luteal function indices.

	Presynch-10 (Control)	PG+G	*P*-values
Cows with functional CL[1], % (n/n)	94 (187/200)	91 (186/204)	0.38
Number of CL[2] ± SEM (n)	2.09 ± 0.07 (169)	2.20 ± 0.07 (173)	0.18
Primiparous	1.98 ± 0.11 (57)	1.95 ± 0.10 (62)	0.90
Multiparous	2.15 ± 0.08 (112)	2.34 ± 0.09 (111)	0.08
Total luteal area, mm^2 ± SEM (n)	712 ± 21 (169)	716 ± 22 (173)	0.91
Primiparous	635 ± 32 (57)	613 ± 33 (62)	0.67
Multiparous	752 ± 27 (112)	774 ± 27 (111)	0.45
Serum P4 at the $PGF_{2\alpha}$ of Ovsynch[2], ng/mL ± SEM	6.14 ± 0.22	5.57 ± 0.19	0.07
Primiparous	6.09 ± 0.34	5.33 ± 0.30	0.11
Multiparous	6.17 ± 0.29	5.70 ± 0.25	0.24

[1]Cows with functional CL = Serum P4 ≥ 1.00 ng/mL.

[2]Only cows with functional CL were used in these analyses.

Table 5.2: Relationships in number of CL, total luteal area, circulating concentrations of P4 and largest follicle diameter at $PGF_{2\alpha}$ of Ovsynch in a subset of first service lactating dairy cows with functional CL (serum P4 ≥ 1.00 ng/mL) at time of $PGF_{2\alpha}$ of Ovsynch (n = 342).

CL number	n	Total luteal area, mm^2 ± SEM	Serum P4, ng/mL ± SEM	Largest follicle diameter[1], mm ± SEM	Percentage of cows within treatments		
					Presynch-10 (control), %	PG+G, %	*P*-value
1	79	398 ± 15 [a]	4.18 ± 0.26 [a]	15.8 ± 0.4 [a]	25 [a]	21 [a*]	0.45
2	166	713 ± 15 [b]	5.89 ± 0.20 [b]	14.3 ± 0.2 [b]	50 [b]	47 [b]	0.52
≥ 3	97	974 ± 23 [c]	7.12 ± 0.31 [c]	13.4 ± 0.3 [c]	25 [a]	32 [a*]	0.15

[1]Only follicles with diameter ≥ 9 mm and < 25 were used in this analysis.

[a-c] Means and percentages within a column with different letters differ ($P < 0.05$).

*Percentages within a column with asterisk had a tendency to differ ($P = 0.06$).

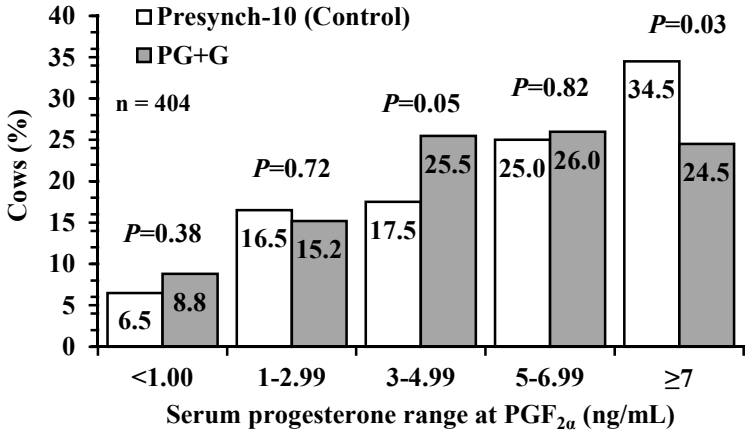

Figure 5.2. Distribution (% of total) of cows within treatments, Presynch-10 (control; n = 200) vs. PG+G (n = 204), across ranges of progesterone (P4) concentrations (ng/mL) at time of $PGF_{2\alpha}$ of Ovsynch.

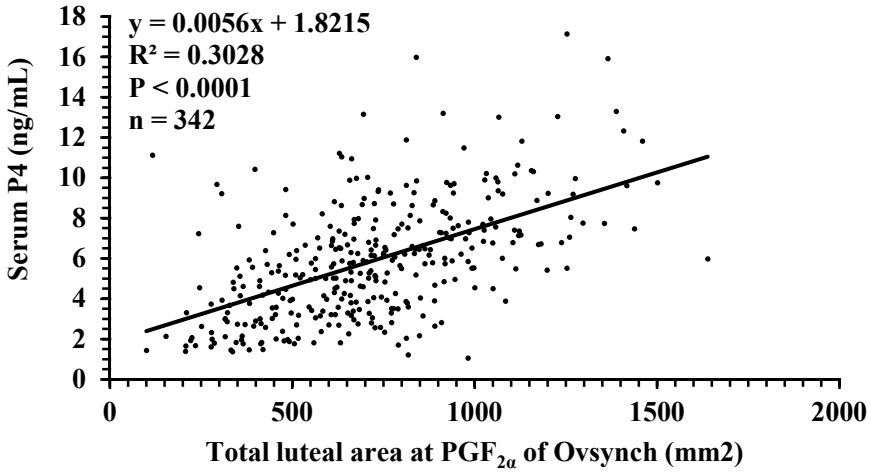

Figure 5.3. Relationship between total luteal area (mm^2) and serum concentrations of progesterone (P4, ng/mL) at time of first $PGF_{2\alpha}$ of Ovsynch in cows with functional corpus luteum (P4 ≥ 1.00 ng/mL).

There was a treatment x parity interaction in mean diameter of the largest follicle at time of first $PGF_{2\alpha}$ of Ovsynch (Figure 8.4). Percentage of cows within treatments that had the presence of a cyst (≥ 25 mm) was not different (P = 0.64) between treatments.

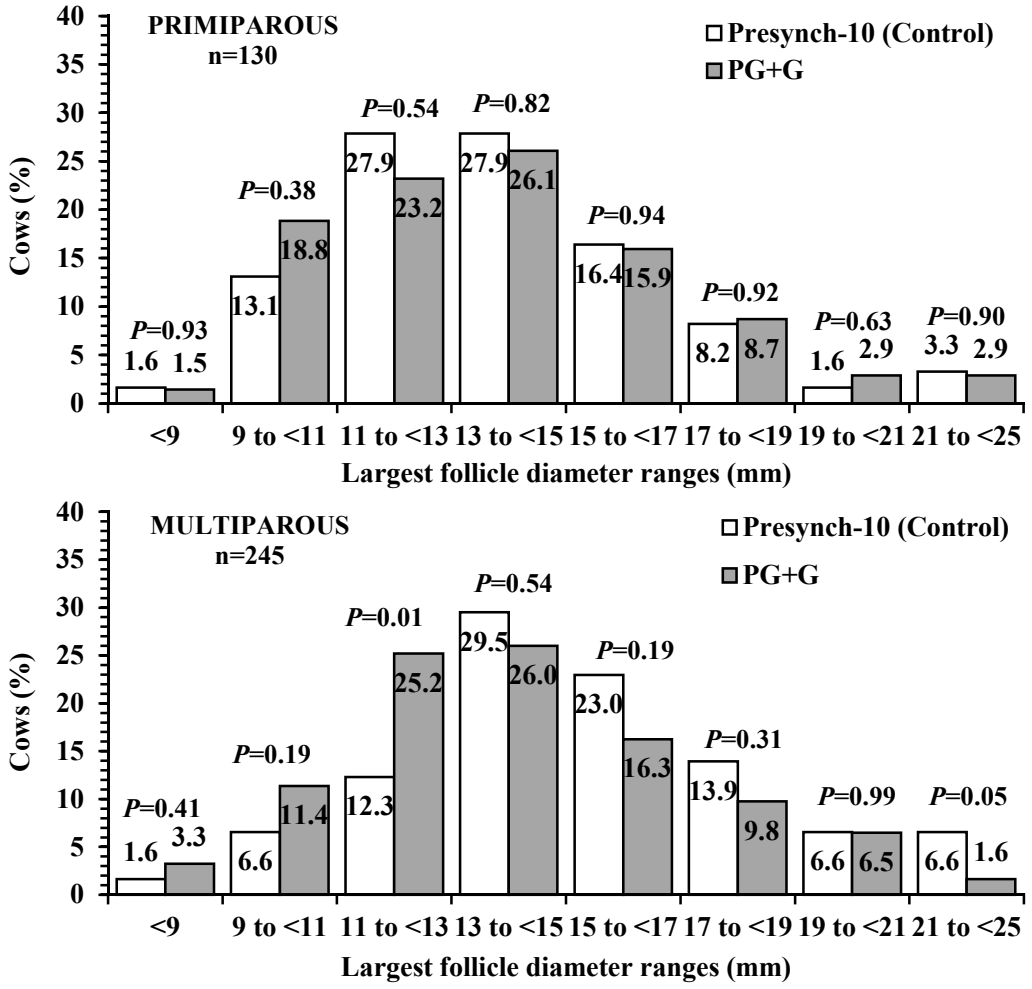

Figure 5.4. Distribution (% of total) of cows within treatments, Presynch-10 (control) vs. PG+G, across different ranges of largest follicle diameter (mm) at time of $PGF_{2\alpha}$ of Ovsynch for primiparous and multiparous cows.

Treatment did not affect P/AI on 28, 35, 49 and 77 d post-AI (Figure 8.5) compared to controls. No difference between primiparous and multiparous was observed on P/AI at 28 (P = 0.16; 52 % vs. 45 %, respectively), 35 (P = 0.17; 48 % vs. 41 %, respectively), 49 (P = 0.11, 45 % vs. 37 %, respectively) and 77 d post-AI (P = 0.13, 43 % vs. 36 %, respectively). Interaction between treatment and parity was not significant ($P \geq 0.59$) for P/AI at any d post-AI. Percentage of cows with pregnancy loss between 28 and 35, 35 and 49, and 49 and 77 d post-AI was not

different between treatments (Figure 8.6). There was no relationship ($P \geq 0.46$) parity and pregnancy loss in any of these periods.

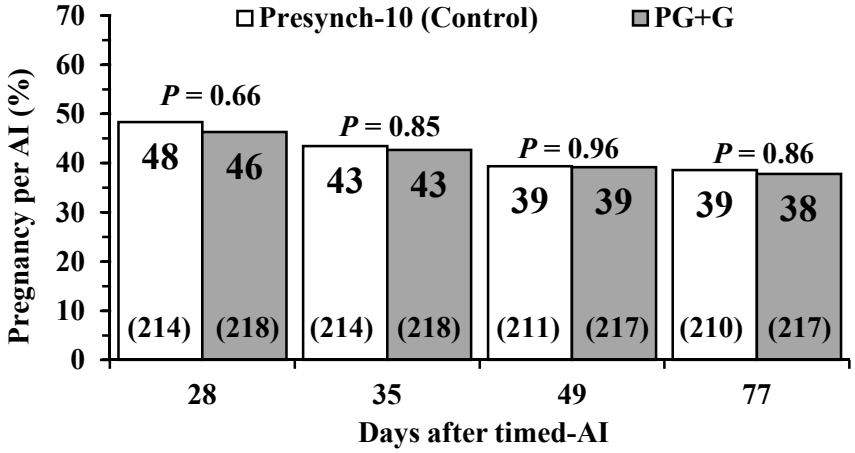

Figure 5.5. Effect of treatment, Presynch-10 (control) vs. PG+G, on pregnancy/AI on d 28, 35, 49 and 77 post-AI.

Cows with pregnancy loss between 28 to 77 d post-AI had similar largest follicle diameters ($P = 0.60$; 14.1 ± 0.6 vs. 14.4 ± 0.2 mm) and serum P4 at time of first $PGF_{2\alpha}$ of Ovsynch ($P = 0.15$; 6.33 ± 0.53 vs. 5.54 ± 0.23 ng/mL) and 28 d post-AI ($P = 0.15$; 7.27 ± 0.52 vs. 7.40 ± 0.20 ng/mL) compared to cows without pregnancy loss during the same period.

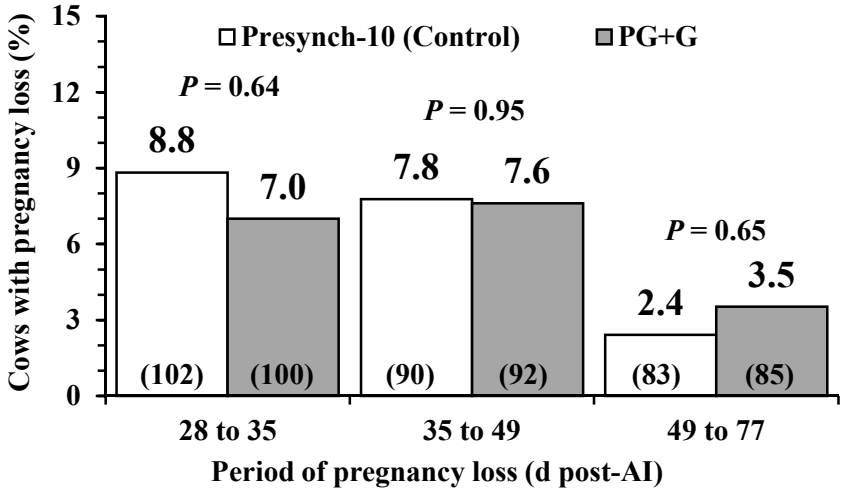

Figure 5.6. Effect of treatment, Presynch-10 (control) vs. PG+G, on percent of cows with pregnancy loss in different periods of gestation.

DISCUSSION

The overarching objective of this study was to develop a simpler and shorter synchronization program for dairy operations that are unable to utilize programs such as G6G, Presynch-10 or Double Ovsynch due to limitations of labor or other logistical constraints. These three fertility programs increase PR/AI compared to AI following estrus [23] and other synchronization programs [4,9,12] primarily because they synchronize a greater percentage of cows on d 6 or 7 of the estrous cycle to increase the chances of ovulation to the first GnRH of Ovsynch [3,4]. Cows that ovulate to the 1st GnRH of Ovsynch have a greater chance for pregnancy [4-6]. The present study tested the simultaneous administration of $PGF_{2\alpha}$ and GnRH 7 d prior to Ovsynch as a pre-synchronization strategy for first service lactating dairy cows.

Pharmacological manipulation of ovarian function to synchronize cows to d 6 or 7 of the estrous cycle requires the synchronous induction of a new estrous cycle. Pre-synchrony administration of $PGF_{2\alpha}$ must induce luteolysis and GnRH must induce ovulation. Fertility programs such as Double Ovsynch, G6G, and Presynch-10 or 11 were designed to maximize the percentage of cows that start Ovsynch on d 6 or 7 of the cycle. No follicle and CL data at time of pre-synchronization treatments or at 1st GnRH of Ovsynch were collected in this study. But, P4 and CL number/size were collected at first $PGF_{2\alpha}$ of Ovsynch. Level of P4 and number of CL at first $PGF_{2\alpha}$ of Ovsynch are key indicators of ovulation to 1st GnRH of Ovsynch and are primarily driven from pre-synchronization outcomes. There was no effect of treatment on number of CL, luteal area or serum concentrations of P4 at time of $PGF_{2\alpha}$ of Ovsynch when compared within parities. Cows with a functional CL at time of $PGF_{2\alpha}$ of Ovsynch had greater chances of pregnancy compared to cows with no functional CL. This is in agreement with a previous study [5]. Both treatments had a high percent of cows with functional CL at time of $PGF_{2\alpha}$ of Ovsynch

(> 90 %). Previous studies that used fertility treatments also reported high percentage of cows with functional CL, including Presynch-10 [87.8 % [24]; 85.7 % [25]], Double-Ovsynch [84.4 % [9], 88 % [10]] and G7G [93.3 % [25]]. Treatment affected percentage of cows with concentrations of P4 in the 3.0 to 4.9 and >7.0 ng/mL. Presynch-10 shifted a greater percentage of cows from 3.0 to 4.9 to > 7.0 ng/mL P4 relative to PG+G.

Pre-synchronization programs that used only $PGF_{2\alpha}$ have been tested to improve fertility of cows treated with Ovsynch by increasing ovulation to the first GnRH of this program. Simple pre-synchronization programs that only used a single injection of $PGF_{2\alpha}$ 3 d [26], 10 d [27] and 12 d [28] prior to Ovsynch did not improve P/AI following timed-AI compared to Ovsynch alone. However, programs that used two $PGF_{2\alpha}$ injections 14 d apart and 14 [15] and 12 d [16,29] prior to Ovsynch were able to improve P/AI compared to Ovsynch without any pre-synchronization. Later, a shorter interval of 11 d between the second $PGF_{2\alpha}$ of the pre-synchronization and Ovsynch (Presynch-11) improved P/AI compared to an interval of 14 d (Presynch-14) [12]. The increase in P/AI for Presynch-11 was attributed to the greater ovulatory response to the first GnRH of Ovsynch for Presynch-11 compared to Presynch-14 [12]. Previous data indicated that the majority of cows that receive two injections of $PGF_{2\alpha}$ 14 d apart are observed in estrus 3, 4 and 5 d following the last $PGF_{2\alpha}$ injection [5,30]. Vasconcelos et al. (1999) showed that the first GnRH of Ovsynch has a greater probability of ovulation when is administered between d 5 and 9 of the estrous cycle. Moreover, data from our laboratory showed that d 6 and 7 of the estrous cycle are the best day of the estrous cycle to induce ovulation following the first GnRH of Ovsynch [4,31]. Therefore, the optimal interval between two $PGF_{2\alpha}$ injections of Ovsynch seems to be 10 (Presynch-10) or 11 d (Presynch-11). Due to this reason

and to the logistics of the reproductive management of the farm, we decided to use Presynch-10 as a control in our experiment to compare with PG+G.

Ribeiro et al. (2012) compared Presynch-10 vs. Double-Ovsynch in combination with two different 5 d Cosynch program (GnRH – 5 d – $PGF_{2\alpha}$ – 1d – $PGF_{2\alpha}$ – 1.5 or 2 d – GnRH + AI) for first service of grazing Holsteins, Jersey and crossbred cows (n = 1,754). There were no differences between Presynch-10 and Double-Ovsynch on P/AI at 30 (59.1 vs. 56.8 %, respectively) and 65 d post-AI (52.4 vs. 52.5 %, respectively). The use of grazing cows with low production (~ 6,000 kg/lactation) and a large number of crossbred cows might explain the greater P/AI achieved in that study [32] compared to our present study. Stevenson et al. (2012) compared Presynch-10 to a variation of the G7G protocol ($PGF_{2\alpha}$ – 3 d – GnRH – 7 d – Ovsynch) in 3,005 dairy cows in 4 different herds and did not find differences in P/AI during the cool-cold weather (October through May). However, G7G had greater P/AI compared to Presynch-10 during the hot season (June through September) [33]. In the present study, PG+G had a 7 d interval between pre-synchronization GnRH and the initiation of Ovsynch as G7G. However, there were no differences on P/AI between PG+G and Presynch-10.

Stevenson (2011) compared Presynch-10 to four different pre-synchronization programs including Presynch-14 and Presynch-12 in combination with a Co-synch program (GnRH – 7d – $PGF_{2\alpha}$ - 72 h – GnRH + AI). In that study, Presynch-10 had similar P/AI at 32 d (35 %) and 60 d post-AI (31 %) compared to Presynch-14 (32 and 29 %) and Presynch-12 (37 and 34 %). In the present study, P/AI of Presynch-10 appears to be greater comparatively. This difference might be due the second injection of $PGF_{2\alpha}$ 1 d after the first $PGF_{2\alpha}$ of Ovsynch [34] and the 16 h interval between the final GnRH of Ovsynch and timed-AI [35] that we used in the present study. Cows that do not have complete luteolysis following the $PGF_{2\alpha}$ of Ovsynch have significantly reduced

chances of pregnancy following timed-AI [36]. The use of an additional $PGF_{2\alpha}$ injection in Ovsynch enhanced the percent of cows with complete luteolysis [34]. And clearly, regarding timing of AI relative to the GnRH-induced LH surge, timed-AI 16 h following the last GnRH of Ovsynch is the optimal time to achieve greater P/AI [35]. Thus, the combination of an extra injection of $PGF_{2\alpha}$ 24 h after the $PGF_{2\alpha}$ of Ovsynch and timed-AI 16 h after the final GnRH of Ovsynch used in our experimental design might optimize P/AI of the Ovsynch program for both treatments. Ensuring luteolysis appears to be critical in any program that utilizes pre-synchrony prior to Ovsynch. In addition, treatment did not change serum concentrations of P4 at time of first $PGF_{2\alpha}$. P4 at time of $PGF_{2\alpha}$ of Ovsynch appears to be a good marker for P/AI. Several studies have shown a positive relationship with P4 at time of PG of Ovsynch and the probability of pregnancy [4,36,37]. The greater the P4 at $PGF_{2\alpha}$ of Ovsynch, the greater chance for pregnancy. PG+G performed well in this regard.

A recent study tested the use of a single injection of GnRH 7 d prior to Ovsynch (GGPG) as a simpler pre-synchronization strategy for first service cows compared to Double-Ovsynch [38]. When used for the resynchronization of non-pregnant cows for second or greater services, GGPG improved P/AI compared to Ovsynch [39-41]. However, first service cows treated with GGPG had reduced P/AI compared to Double-Ovsynch [38]. One of the problems of GGPG for first service pre-synchronization was attributed to the greater percentage of cows in the late stages of the estrous cycle at the beginning of the Ovsynch [38]. A greater percentage of cows treated with GGPG had serum P4 < 2.00 ng/mL at time of $PGF_{2\alpha}$ of Ovsynch. These cows might had started the endogenous luteolysis process prior to the administration of $PGF_{2\alpha}$ of Ovsynch most likely resulting in an LH surge prior to the final GnRH of Ovsynch and ovulation prior to the timed-AI [3,42]. Cows ovulating prior to timed-AI had significantly reduction in P/AI [35].

The difference between GGPG and PG+G used in the present study is the administration of $PGF_{2\alpha}$ simultaneously with the first GnRH of GGPG, 7 d prior to Ovsynch. It appears it is important to initiate a new cycle with any pre-synch program, in this case $PGF_{2\alpha,}$ to avoid initiating Ovsynch too late in the estrous cycle.

Taken together, PG+G could be advantageous because it only adds one more d of animal handling to the Ovsynch program, and it does not add any extra weekdays for injections compared to Ovsynch. The implementation of PG+G might reduce final cost of the pre-synchronization program compared to Presynch-10. Although GnRH products are usually slightly more expensive than $PGF_{2\alpha}$ products, reduction of labor cost with one less weekday to administer injections would reduce the final cost of the program. In addition to the economic advantage, PG+G reduced the length of the program compared to Presynch programs.

CONCLUSIONS

Results from the present study indicate that administering both $PGF_{2\alpha}$ and GnRH on the same day, 7 d prior to the start of Ovsynch (PG+G), can achieve similar P/AI compared to Presynch-10. Percentage of cows with functional CL, serum concentrations of P4 and number and size of CL and follicles at time of $PGF_{2\alpha}$ of Ovsynch appear to be similar to other fertility programs. Thus, PG+G may offer a reasonable alternative to more complex fertility programs to enhance P/AI for first service. This program may be a good strategy for farms that want to limit the number of d in the week cows are handled.

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SECTION 2

EFFECT OF INDUCTING HIGH AND LOW SERUM CONCENTRATIONS OF PROGESTERONE DURING GROWTH OF THE OVULATORY FOLLICLE ON FOLLICLE DEVELOPMENT AND FERTILITY OF LACTATING DAIRY COWS AND DAIRY HEIFERS

CHAPTER 6

REVIEW OF PROGESTERONE FUNCTION DURING OVULATORY FOLLICLE DEVELOPMENT IN CATTLE

OVERVIEW OF FOLLICLE DEVELOPMENT DURING THE ESTROUS CYCLE IN CATTLE

The introduction of the ultrasonography in the study of ovaries in cattle, has enabled the visualization of antral follicles with diameter of ≥ 2 mm daily, during the entire estrous cycle. The use of this instrument, allowed the detection of wave-like patterns of antral follicle growth during the estrous cycle of cows and other monoovulator species. This pattern is characterized by recurrent waves of follicular development during the estrous cycle referred to as "follicular waves" [1,2]. Follicular waves were detected in pre-pubertal heifers [3], pregnant cows [4], during the postpartum period [5], and during anovular periods [6]. However, in these cases, there is no endogenous gonadotropin stimulation for follicles to ovulate (LH surge), and they become atretic. During a typical estrous cycle, cows have 2 to 4 follicular waves, but a 2 wave-pattern is predominant [7]. The length of a typical estrous cycle in a high producing lactating cow is in average 23 days [7] but it can range from 19 to 25 d (Figure 1.1A). Each follicular wave is initiated with the cyclic recruitment [8], which is the emergence of a cohort of multiple small follicles (2 to 3 mm in diameter). A wave ends with atresia or ovulation of a single dominant follicle (DF), which in this case is the ovulatory follicle [9]. The fate of the DF is dependent of the phase of the estrous cycle. The DF that develops during the luteal phase, ultimately undergoes atresia after losing its E2-producing capacity. On the other hand, the DF that develops during the follicular phase ovulates. The majority of follicles (99.9%) become atretic by apoptosis, only few ovulate [10]. The process of follicle atresia is fundamental to regulate: (1) the formation of a single dominant follicle from a group of growing follicles and (2) the lifespan of the dominant follicle in non-ovulatory follicular waves. Antral follicles that undergo atresia lose their estradiol producing capacity, and decrease the follicular fluid estradiol to progesterone ratio [11-13]. Thus, estradiol synthesis capacity is an indicator of the follicle health condition.

Therefore, understanding the mechanisms that control estradiol production, stimulation, and suppression, can help us elucidate how a single dominant follicle is selected from a cohort.

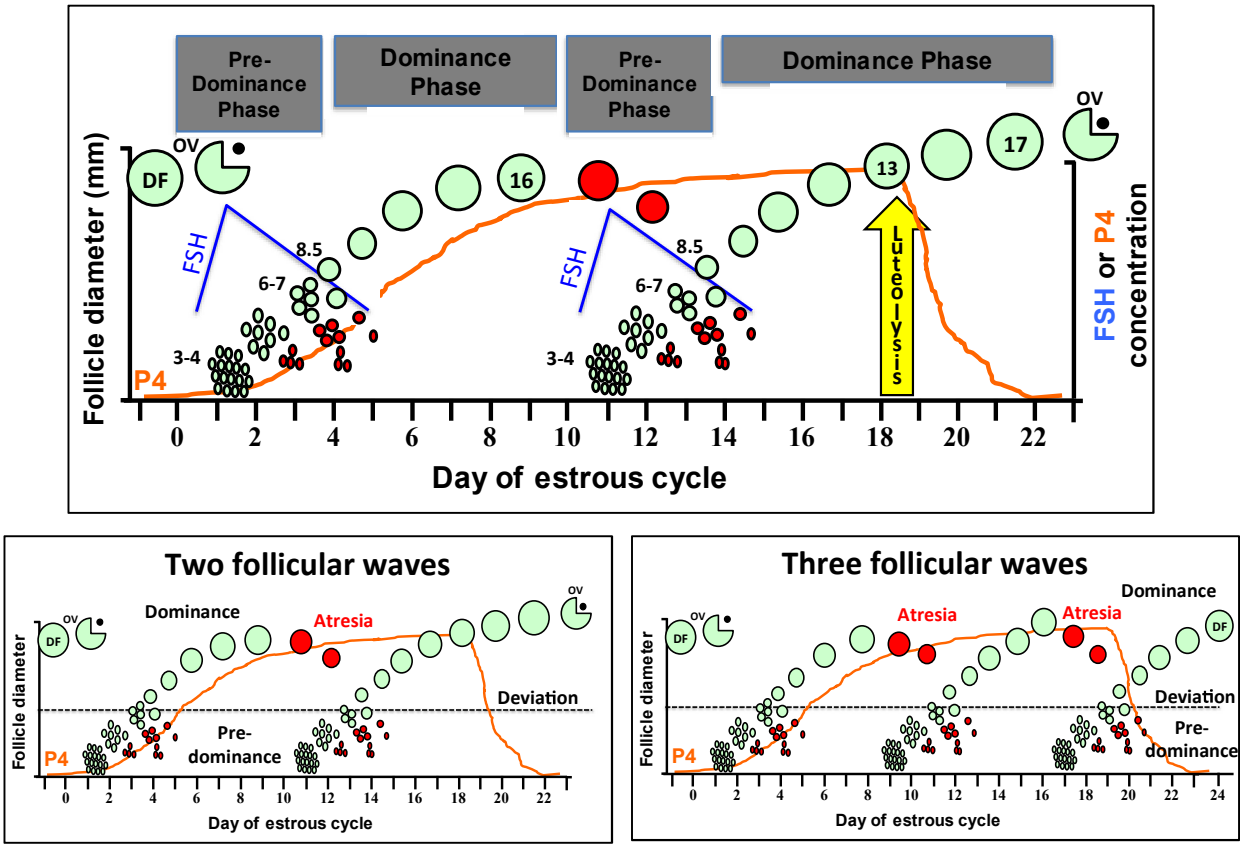

Figure 6.1. A: Schematic description of the pattern of secretion of follicle stimulating hormone (FSH) and progesterone (P4) and follicle growth during a typical estrous cycle of a lactating dairy cow with 2 follicular wave based on recent data. **B and C:** Schematic description of the pattern of serum progesterone (P4) and follicle growth during a typical estrous cycle of a lactating dairy cow with 2 (B) and 3 (C) follicular waves. (Health growing follicles= green / atretic follicle= red / OV= ovulation).

In cattle, the first follicular wave of the estrous cycle starts on d 0 = day of estrous cycle. In cows with two-wave patterns, length of the first wave is about 8 to 14 d, with the second wave (ovulatory wave) starting approximately mid-cycle [7] (Figure 1.1B). However, in a three-wave cow, wave duration is shorter, 7 to 8 d for each wave. The second wave starts around d 8 and 9 of the cycle, and the third wave (ovulatory wave) starts around d 14 to 17 of the cycle [7,14]

(Figure 1.1C). In cows with two waves, the dominant follicle (DF) of the second wave is the ovulatory follicle, while in a three-wave cow the DF from the third wave is the ovulatory follicle. Cows with three waves have approximately 2 d longer cycle when compared to cows with two waves.

During the follicular wave of cows, one of the follicles from the cohort grows from a small antral diameter of approximately 2 mm to large antrum stage, avoids atresia and becomes a dominant follicle with diameter between 10 to 20 mm [7]. This is an increase up to 10-fold in follicle diameter, with a significant increase in overall volume. This increase in size is a result of antrum expansion and, in part, of the proliferation of granulosa cells. During the follicular wave, follicular steroid activity and response to gonadotropins are tightly controlled to: (1) prevent premature luteinization during the fast follicular growth; (2) induce a select cohort of follicles for further growth; (3) regulate atresia of subordinate follicles and selection of a dominant follicle. In order to achieve these aims, different levels of regulation, i.e. autocrine, paracrine and endocrine, and several different factors, that exhibit spatiotemporal expression patterns, are in action. Some of these factors include growth factors locally produced derived from granulosa cells such as activin and inhibin, the oocyte such as GDF-9 and BMP-15, and theca cells such as androgen (Figure 1.2). Theca and granulosa cells have significant differential gene expression during different stages of follicular wave (> 7,500 for each cell type) [15]. A 1.5 fold change was detected during progress from small (<5 mm) to medium (5-10 mm), medium to large (>10 mm), or small to large [15].

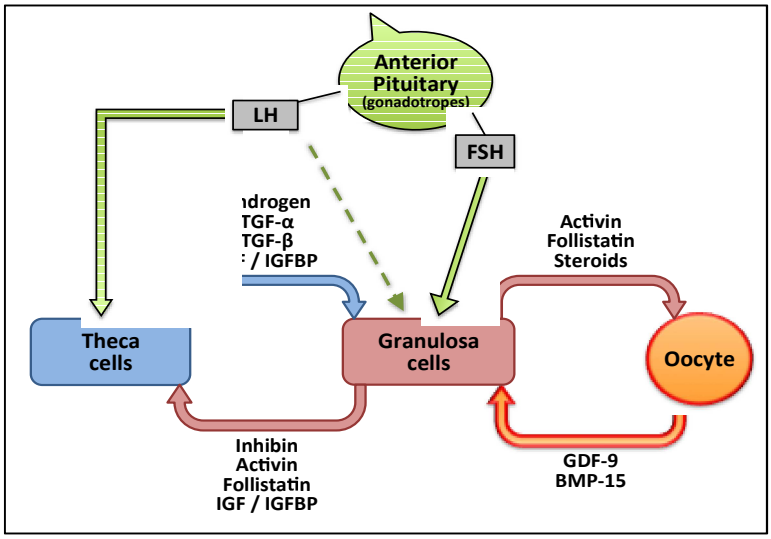

Figure 6.2. Simplified schematic description of the 3 main cell types of antral follicles with interaction of gonadotropin hormones and intrafollicular factors likely to be involved in communications between theca cells, granulosa cells and the oocyte. BMP-15: bone morphogenetic protein 15; GDF-9: growth and differentiation factor 9; IGF: insulin-like growth factor; IGFBP: insulin-like growth factor-binding proteins.

Recruitment

The follicular wave is subdivided in three different phases: recruitment (cyclic), selection, and dominance [9] (Figure 1.3). Recruitment is initiated by the emergence of the cohort of follicles that is generated by a transient increase in follicle stimulating hormone (FSH) [16-18]. Recruitment marks the initiation of each follicular wave and is part of the pre-dominance phase. The initial increase in concentrations of FSH during recruitment, is a result of the loss of negative feedback from inhibin and E2 after atresia or ovulation of the DF from the previous wave. During the recruitment period, follicle growth is dependent on FSH acting upon FSH receptors (FSH-R) located in granulosa cells [18-20], promoting cellular growth and proliferation of granulosa cells [21]. This mitogenic action is direct in granulosa cell receptors by induction of cyclin-dependent kinases (CDKs) and interaction with epidermal growth factors (EGF) [22]. An

indirect mitogenic action of FSH in granulosa cells is via the production of estradiol, which in turn induce cell proliferation by different signaling pathways [23].

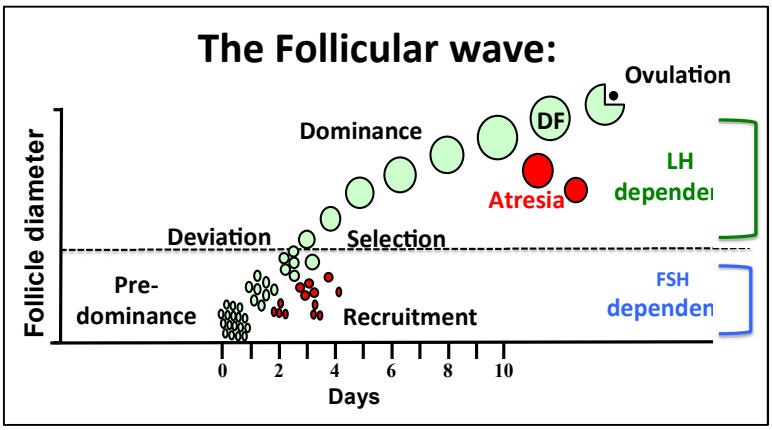

Figure 6.3. Illustration of the phases of the follicular wave in cattle.

The transient increase in FSH induces expression of cytochrome P450 aromatase (CYP19A) in granulosa cells, which is responsible for the conversion of androgen to estradiol [24]. Androgen substrate for estradiol production is synthesized by theca cells via cell-specific expression of cytochrome P450 17α (CYP17A), which is induced by LH receptors [25]. Granulosa cells lack CYP17A, and, therefore, are unable to produce androgen for E2 production. Thus, the collaborative relationship between granulosa and theca cells that require the actions of FSH and LH. This concept is termed the two-cell, two-gonadotropin model (Figure 4) [26]. E2 appears to act via estrogen receptor (ER) in granulosa cells to enhance overall FSH actions [27]. The production of E2 and inhibin increases as follicles from the cohort increase in size during recruitment, increasing the negative feedback for FSH on the anterior pituitary gland. The majority of antral follicles undergo atresia due to insufficient gonadotropin support, but some antral follicles avoid atresia, and increase in size after emergence. These antral follicles have an approximate similar growth rate (common growth phase) and each follicle has the ability to become dominant [2]. However, the largest follicle is the one that usually becomes dominant [1].

The moment when the two largest follicles from the common growth phase diverge in growth rates and E2-producing capacity is termed deviation, which characterizes the end of the selection phase. Studies that used follicle ablation (aspiration) of the largest follicle at time of deviation showed that other follicles from the cohort retain the capacity to become dominant [2].

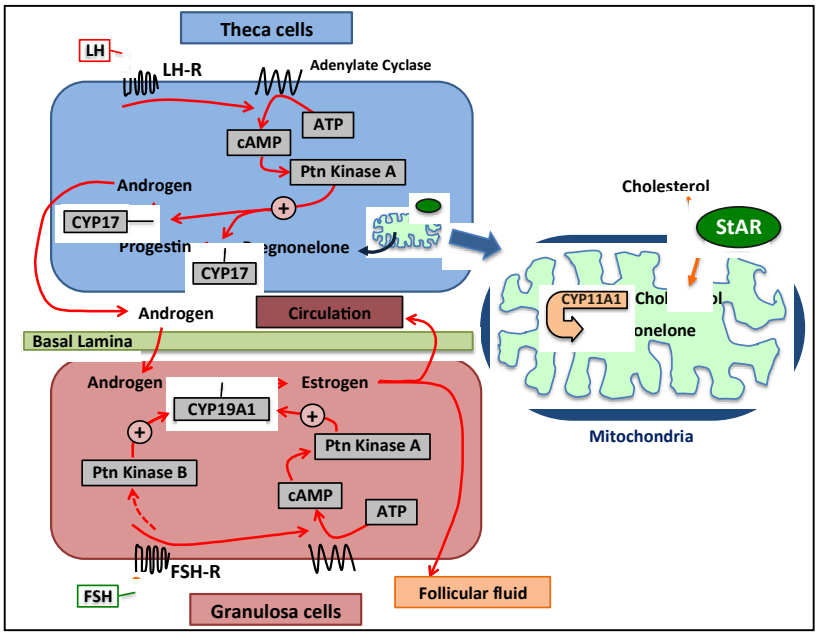

Figure 6.4. Diagram of the two-cell, two-gonadotropin system for estradiol synthesis in the follicle. Modified from Erickson GF and Shimasaki (2001) [26].

Selection and dominance

At the beginning of deviation, approximately 60 h after emergence, the largest follicle reaches 8.5 mm of diameter and greater amounts of E2 when compared to the rest of the follicles within the cohort [28]. The remaining subordinate follicles cease E2 production and stop growth. The intrafollicular mechanisms that lead to the selection of a dominant follicle, start prior to deviation and occur in parallel with a decline in FSH concentrations. The decrease in FSH is associated with an increase in E2 and inhibin concentrations in the follicular fluid of the larger follicle, which enhance a negative feedback on the anterior pituitary gland [24] (Figure 5). Granulosa

cells produce inhibin that regulates FSH production in the pituitary. Inhibin antagonizes the actions of activin on FSH secretion, and reduces gonadotropin sensitivity to GnRH receptors [29]. Inhibin may also enhance E2 production of follicles. Studies using culture of bovine theca cells in vitro showed that inhibin increases LH-stimulated and E2-stimulated production of androgens [30]. In this system, activin had the opposite effect, inhibiting LH- and E2-stimulated androgen production by theca cells. In addition, activin reversed the positive effect of inhibin when combined in the same system. Follistatin that is a high-affinity activin-binding protein neutralized the effects of activin [30]. This study indicated that these members of the TGF- β superfamily (activin, inhibin and follistatin) produced by granulosa cells, might have a paracrine role in modulating the production of androgens by theca cells.

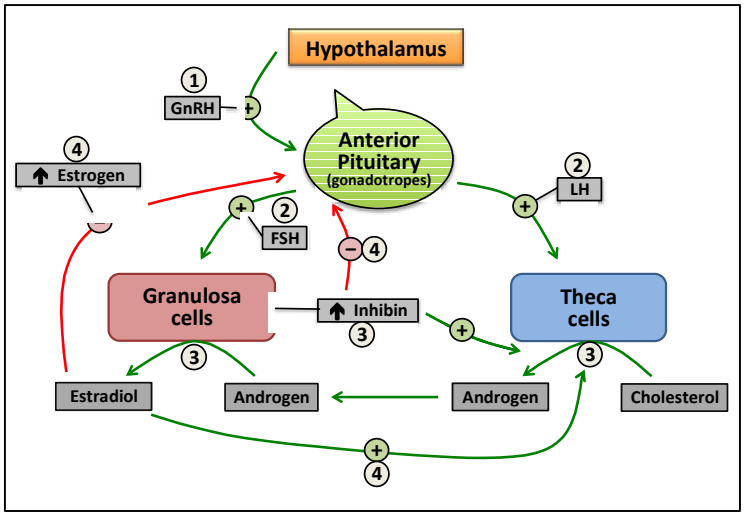

Figure 6.5. Schematic diagram summarizing potential intrafollicular roles of inhibin and estrogen. (1) With lack of negative feedback, GnRH pulses induce release of gonadotropins (LH and FSH) that will act on their target cell (2). LH induces production of androgens by theca cells, and FSH stimulates synthesis of estradiol from androgen and inhibin (3). (4) Increased concentrations of estradiol and inhibin causes a negative feedback on FSH secretion from the anterior pituitary.

Insulin growth factors (IGF) and their binding proteins (IGFBPs) are also regulators of follicle selection and atresia. During the selection process insulin-like growth factor 1 (IGF-1) bioavailability (free IGF-1) increases in the follicular fluid of the DF, which is associated with an increase in E2 production, and proliferation and differentiation of granulosa cells [31,32]. IGF-1 also stimulates inhibin during the follicular phase of ewes in vivo (Campbell 1988). The bioavailability of IGF-1 is regulated by binding proteins and proteases (insulin-like growth factor binding protein 1-6: IGFBP 1-6, and pregnancy-associated plasma protein-A: PAPP-A) [33]. IGFBPs are present in the follicular fluid and bind to IGFs, inhibiting their actions, while PAPP-A is the main proteolytic enzyme that degrades IGFBPs. Concentrations of IGFBP-2, 4 and 5 are greater in subordinate follicles compared to the DF [34]. These results are in agreement with other studies that indicated a greater concentration of free IGF-1 in the follicular fluid of the DF when compared to subordinate follicles [2,35], and a greater amount of PAPP-A mRNA in the DF than in subordinate follicles [36]. A recent study investigated the composition of follicular fluid of medium size follicles prior to deviation, and followed their fate. Mihm et al. [37] aspirated a small amount of follicular fluid in vivo of three follicles on d 3 of the estrous cycle and followed their development daily by ultrasonography examinations of the ovary. The aspirated follicles were not atretic at time of collection because all of them were producing estradiol. However, the follicles that became dominant, had greater estradiol concentrations and lower concentrations of IGFBP-4 in follicular fluid when compared to subordinate follicles 1.5 d after emergence. Therefore, changes in free IGF-1 might occur prior to the loss of E2-producing capacity of subordinate follicles.

Recently, a new factor that has been associated to the selection process in cattle was the cocaine and amphetamine regulated transcript protein (CARTPT) [38]. In random stages of the

first follicular wave, atretic estrogen-inactive subordinate follicles had greater levels of CARTPT mRNA and follicular fluid CARTPT peptide than estrogen-active DFs [39]. Levels of CARTPT mRNAS in DF decreased right after selection, indicating a potential role of this factor during this process. CARTPT mRNA was also greater in subordinate follicles compared to dominant follicles prior, and right after, deviation of the dominant follicle [38]. Experiments using the mature form of the CARTPT peptide (CART) in vitro and in vivo, determined that CART has a potential inhibitory effect on granulosa cell E2 production. Both FSH- and IGF-1-stimulated estradiol production are negatively affected by CART [38,40]. These effects are caused by inhibition of components of the FSH signaling pathway, resulting in a decrease in cAMP production and Ca^{2+} influx and, consequently, a reduction in CYP19A1 [38,40,41]. Additionally to the effect on granulosa cells, CART also reduced synthesis of LH-induced androstenedione by theca cells in vitro [42]. Taken together, strong evidence supports the role of CART as a main regulator of atresia during the selection of the dominant follicle in cattle.

Two important events occur close to deviation of the DF and are related to the selection of the DF. First, granulosa cells of the DF express LH receptors (LH-R). Simultaneously, a temporary increase in circulating LH enhances the response of the DF to LH. Increase in production of E2 in granulosa cells of the DF may be related with the acquisition of LH-R in granulosa cells during deviation. Estradiol enhances the differentiation of granulosa cells (LH-R expression) induced by FSH in mice. This E2 action appears to be via cAMP pathway [43]. Expression of LH receptors in the granulosa cells increases expression of CYP19A1 and enhances secretion of estradiol [16]. As a result, the DF continues to grow and concentrations of FSH decrease to nadir levels, which are lower than those required by subordinate follicles to grow. This also helps maintain the dominance of the DF. After deviation and acquirement of LH-

R by the granulosa cells, the follicle switches its dependency from FSH to LH, and it is capable of ovulation. During the estrous cycle, a DF is capable of ovulating if luteolysis occurs prior to its atresia.

The duration of the dominance phase depends on the pulse frequency of LH to support follicle growth and E2 production. Progesterone exerts a negative feedback on the GnRH neurons of the hypothalamus, reducing basal GnRH frequency and amplitude, which reduces the frequency of LH pulses. Enhanced production of E2 by the DF, combined with low P4 after luteolysis, induce a positive feedback to the hypothalamus to release a surge of GnRH and consequently the pre-ovulatory LH surge. The LH surge stimulates follicular cell modifications, cumulus expansion, oocyte maturation prior to the ovulation, and degradation of the follicular wall to facilitate the extrusion of a mature oocyte. Following the LH surge and ovulation, follicular cells transform into luteal cells in a process that involves morphological, endocrinological and biochemical modifications [44]. This process generates a vascular corpus luteum with high progesterone producing capacity to sustain a potential pregnancy.

EFFECTS OF LOW P4 ON DURATION OF DOMINANCE AND FERTILITY

The duration of the dominance phase depends on the pulse frequency of LH to support follicle growth and E2 production. Progesterone exerts a negative feedback on the GnRH neurons of the hypothalamus, reducing basal GnRH frequency and amplitude, which reduces the frequency of LH pulses. Thus, cows and heifers with high concentrations of P4 have enhanced negative feedback and lower pulse frequency of LH, and cows and heifers with low concentrations of progesterone have reduced negative feedback and greater pulse frequency of LH. Low circulating concentrations of P4 for a prolonged period during dominance promotes

continuous growth of the dominant follicle in dairy heifers and cows, resulting in a longer duration of dominance and a greater dominant follicle size. In addition, time from emergence to atresia of the DF of the first wave was longer, delaying the emergence of the second wave for heifers treated with low P4 compared with high P4. In contrast, DF of heifers treated with high serum concentrations of P4 had earlier atresia compared with heifers treated with low P4. Cows and heifers with sub-luteal circulating concentrations of P4 (1-2 ng/mL, low P4) exhibit a prolonged dominance phase. Follicles with prolonged dominance phase are referred to as persistent follicles. Persistent follicles have a longer lifespan than a normal dominant follicle. Although pulses of LH are necessary for normal follicular development, increased pulse frequency of LH, created by low circulating levels of P4 during a prolonged period of dominance, were associated with infertility in cattle [45-47]. Heifers inseminated after ovulation of persistent follicles had lower conception rates compared with heifers having ovulatory follicles with a reduced dominance period. Oocytes from persistent follicles undergo premature resumption of meiosis [48] and early germinal vesicle breakdown [45]. As a result, the oocyte from a persistent follicle may be at a more advanced stage of maturation than those from dominant follicles of normal age and size by the time of ovulation. This aged oocyte has lower fertility, resulting in high early embryonic mortality. These data determined that low progesterone environment, and high pulses of luteinizing hormone for an extended dominance period, are detrimental for the oocyte competence. However, it is unclear if the detrimental effects of low P4 for a prolong duration of dominance would also be found in follicles induced with low P4 for a short duration of dominance. Therefore, controlling the duration of dominance of the ovulatory follicular wave and oocyte age, is fundamental to test the effect of circulating concentrations of P4 during the growth of the ovulatory follicle on fertility. The experimental

design of the study in Chapter 3 controlled ovulatory follicular wave duration prior testing the effect of low vs. high circulating P4 on fertility of lactating dairy cows.

FACTORS THAT IMPACT SERUM CONCENTRATIONS OF PROGESTERONE DURING THE ESTROUS CYCLE OF LACTATING DAIRY COWS

Progesterone is a steroid hormone that during the estrous cycle in cattle is synthesized and secreted by the corpus luteum (CL). The CL is formed by luteinization of follicular cells of the ovulatory follicle, endothelial cell invasion, and tissue remodeling following ovulation [49]. Circulating concentrations of P4 during the luteal phase is positively related to the volume of luteal tissue [50], capacity of the luteal tissue to synthesize P4 and blood flow of the CL. The amount of luteal tissue is dependent on the number and size of luteal cells that is dependent on size (volume), number, and age of the CL. Furthermore, the size of the CL is positively correlated to the size of the ovulatory follicle that formed the CL. Despite of the fact that lactating dairy cows have greater CL compared to heifers, peak serum concentrations of P4 in lactating dairy cows are lower compared to heifers [7,14,51].

Researchers in Wisconsin [52] investigated the hypothesis that elevated feed intake increase liver blood flow, resulting in an increase of steroid metabolic clearance rate (MCR). This hypothesis was driven by previous research in sheep and pigs that demonstrated an inverse relationship between circulating P4 and feed intake. Another factor that motivated this hypothesis was the decrease in reproductive efficiency of high producing lactating dairy cows in the last decades, that was associated with the increase in milk production and dry matter intake during the same time. The theory was that lactating cows require greater dry matter intakes to produce high yields of milk, resulting in an increased blood flow passing through the liver, and greater steroid metabolism. Continuous infusion of P4 and E2 in the bloodstream was used to

reach a steady state circulating concentration [52]. The study determined that liver blood flow and steroid metabolism were significant increased by feed consumption in lactating and non-lactating cows. In addition, lactating cows had greater liver blood flow and steroid MCR compared to non-lactating cows.

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CHAPTER 7

IMPACT OF CIRCULATING CONCENTRATIONS OF PROGESTERONE AND ANTRAL AGE OF THE OVULATORY FOLLICLE ON FERTILITY OF HIGH-PRODUCING LACTATING DAIRY COWS

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ABSTRACT

Dairy cow infertility negatively affects profit of dairy production enterprises around the world, and enhancing conception rates of dairy cows is a critical management issue to resolve. It appears that conception rates of dairy cows are attenuated due to reduced progesterone concentrations in circulation during growth of the ovulatory follicle. It is not clear how reduced progesterone influences fertility, but data presented in this brief review suggest that it can be somewhat reversed through increasing concentrations of progesterone during the growth of the ovulatory follicle before luteolysis. Ovsynch protocols may be utilized to enhance progesterone concentrations through the induction of an accessory corpus luteum (CL) following the initial gonadotropin-releasing hormone (GnRH) treatment. Cows at Day 13 of the estrous cycle with a 7-day-old accessory CL had ~ 50% more progesterone at the time of prostaglandin injection of Ovsynch compared with cows with only a Day 13 CL. Ovsynch can consistently induce an accessory CL following the initial GnRH treatment if cows are on Days 6 or 7 of the estrous cycle at the time of treatment. Pre-synchrony strategies are critical to enhance the probability that cows will be on Days 6 or 7 at first GnRH treatment of Ovsynch.

INTRODUCTION

Infertility of the lactating dairy cow continues to be a critical problem limiting profitability and sustainability of dairy farms [1,2]. Reproductive performance of lactating dairy cows is dependent on service rate (or estrus detection rate), fertility of the service sire and maternal fertility. Service rate can be controlled utilizing gonadotropin-releasing hormone (GnRH)-based (Ovsynch) technology (Figure 2.1; [3,4]). The majority of dairy producers in the USA regulate time to first and subsequent artificial inseminations (AI) with Ovsynch technology [5]. High fertility sires can be chosen utilizing the USDA-ARS sire conception rate summaries.

Yet maternal fertility, defined as the mother's ability to ovulate a competent oocyte and provide an oviductal and uterine environment capable of fertilization and complete embryonic and fetal development, continues to be the key limiting factor for profitable reproductive performance in lactating dairy cows.

Conception rates of lactating dairy cows are ~ 30% [6] compared with 60% in virgin dairy heifers [7,8] when inseminated following a detected estrus. Increasing conception rates of lactating cows to that of heifers would allow producers to employ the most profitable calving interval strategies for cows with different production levels, and increase profit. Aspects of maternal fertility that limit conception and embryonic/fetal development are not well understood. Yet it appears that Ovsynch technology may be able to be modified to address these issues. A significant change in circulating concentrations of steroid hormones takes place following the transition from heifer to lactating dairy cow. Circulating concentrations of progesterone (P4) in lactating dairy cows are nearly half the levels detected in nulliparous heifers [9]. This difference in circulating P4 appears to influence follicle growth by prolonging the age of the ovulatory follicle via reduced negative feedback of P4 on pulses of luteinizing hormone (LH; [10]). Allowing more LH pulses to occur may drive the growth of a dominant follicle (DF) similarly, but likely not to the extreme, as with a persistent follicle. Oocytes from this potentially LH over-stimulated ovulatory follicle may have similar characteristics to a persistent follicle and may be less competent to fertilize and/or develop into a competent embryo compared with oocytes from animals with greater concentrations of P4 [11].

This paper will focus on how increasing circulating concentrations of P4 before prostaglandin $F_{2\alpha}$ (PGF)-induced luteolysis during Ovsynch may significantly enhance fertility of lactating dairy cows. Our data strongly suggest that initiating Ovsynch on Days 6 or 7 of the

estrous cycle induces ovulation to the first GnRH more than 90% of the time and induces an accessory corpus luteum (CL), thereby increasing concentrations of P4 before PGF-induced luteolysis 7 days later [12]. Inducing ovulation and a new accessory CL also induces a new follicular wave and the growth of a new DF. This allows for greater control of ovulation of a young, yet mature ovulatory follicle.

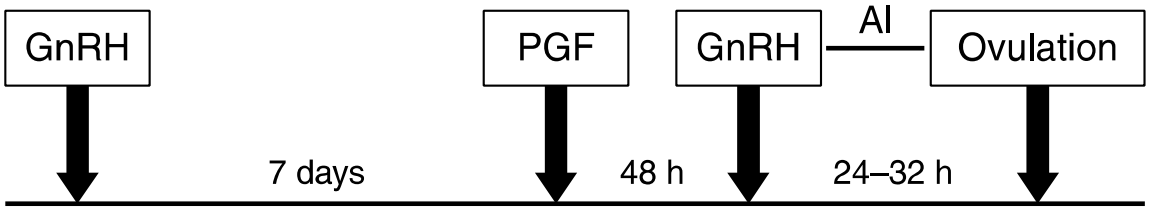

Figure 7.1. Description of the original Ovsynch program utilizing GnRH and prostaglandin $F_{2\alpha}$ (PGF) to control the time of ovulation in lactating dairy cows. AI, artificial insemination.

EFFECT OF LOW P4 ON OVARIAN DYNAMICS AND FERTILITY OF LACTATING DAIRY COWS

The problem

During the past 50 years, conception rates of lactating dairy cows in the USA have decreased from ~ 60% [13,14] to 30% [15,16], while conception rates in heifers remained steady at ~ 60% [7,14]. During that time milk production per cow and dry matter intake have increased linearly [2]. Published data now indicate that high dry matter intake in high-producing dairy cows results in increased blood flow to the liver and increased steroid metabolism [17]. Even though lactating dairy cows have larger ovulatory follicles and CL than nulliparous heifers, circulating concentrations of estradiol (E2) and P4 are significantly less in cows compared with heifers [18]. In addition, there are clearly greater concentrations of E2 and P4 in mature beef cows compared with mature lactating dairy cows [19]. This difference can only be explained by differences in

level of milk production and dry matter intake. Thus, the modern high-producing, lactating dairy cow is deficient in E2 and P4. This deficiency may be partly responsible for the decline in conception rates.

Evidence that low P4 may be responsible for the decline in conception rates of lactating dairy cows

Treatments that maintain low concentrations of P4 (1–2 ng/mL) for an extended period cause prolonged growth and dominance of the DF, and can be made to persist longer than the lifespan of a normal DF [20-24]. When the persistent DF is allowed to ovulate, fertility is decreased in comparison with younger ovulatory follicles [21,23,25-27]. Sub-luteal levels of P4 during a prolonged period increased frequency of LH pulsatility [28,29]. As a result, the oocyte housed in this persistent DF may resume meiosis while still contained in the follicle and could undergo premature nuclear maturation. Histological characteristics of these persistent DFs indicate that the oocyte undergoes early germinal vesicle breakdown and continues a progression through the cell cycle towards metaphase I or II [27,30]. By the time of ovulation of the persistent follicle the oocyte has already matured and aged resulting in reduced fertility, which may be explained either by low fertilization rates [31,32], high early embryonic mortality [33] or both. In a normal estrous cycle, lactating dairy cows have about half the circulating concentrations of P4 to that of heifers [9]. We hypothesize that follicles that grow in this environment may have similar characteristics to persistent follicles that grow under slightly lower concentrations of P4. Thus, increasing P4 by utilizing Ovsynch technology to induce an accessory CL may resolve this problem. Our data indicate that, compared with a normal estrous cycle, inducing an accessory CL during Ovsynch increases serum P4 from 3.5 to 5.2 ng/mL, which increases the likelihood that the ovulatory follicle will emerge (J. R. Pursley, unpubl. data), deviate from subordinates and

develop into a DF. These data indicate that the greater the P4 before PGF-induced luteolysis, the greater the conception rates.

OVERVIEW OF OVSYNCH

This overview of Ovsynch intended to describe the potential outcomes of each injection of hormone within the Ovsynch protocol and the reasons for each outcome. It provides the basis for why cows must be on Day 6 or 7 of the estrous cycle when Ovsynch is initiated, and thus why an efficient pre-synchronization program is essential to achieve the goal to induce accessory CL and increase P4 before PGF-induced luteolysis (Figure 2.2). Ovsynch is based on three treatments: the first treatment, 100 mg of GnRH, in absence of pre-synchrony programs, is administered at a random stage of the estrous cycle (Day 0). The intent of the first GnRH-induced LH surge is to induce ovulation of a mature functional DF [3]. Cows with two follicular waves have an approximately 70% chance that a functional DF capable of ovulation is present in the ovaries at a random stage of the estrous cycle. Ovulation of the DF induces the subsequent emergence of a new follicular wave ~ 1.5 days later [3] followed by the growth and development of both a new DF and an accessory CL during the next 7 days. The new DF has an approximately 97% chance of remaining functional during the 7 days leading up to the PGF injection on Day 7, then continuing on to ovulation following the final GnRH-induced LH surge on Day 9, even if luteolysis following the PG is not complete [3]. At random stages of the estrous cycle, cows have an ~ 30% chance of being in early stages of follicular development (first or second wave) at a time when granulosa cells have not yet acquired sufficient LH receptors to respond to an LH surge [34]. In this case, the first GnRH does not induce ovulation and the potential DF continues to grow, deviate from subordinates and develop as a DF [3]. This DF has an approximately 80%

chance of remaining functional before the PGF-induced luteolysis and subsequent increase in LH pulsatility that allows further development [3]. If this DF remains functional until the time of PGF injection, it will continue to develop into a pre-ovulatory follicle and has a 97% chance of ovulation following the final injection of GnRH [12]. However, this follicle could be as much as 12 days from emergence. Thus, conception rates following the ovulation of this follicle could be attenuated due to the antral age of the follicle and oocyte. Approximately 20% of these follicles become atretic before the PGF injection. If this happens, a new wave will emerge generally just before the PGF and the largest follicle may deviate from subordinates, but this follicle may not have deviated from subordinates and acquired LH receptors before the final GnRH-induced LH surge [34]. In this scenario, cows will likely have a natural estrus that may or may not be detected several days after the timed-AI as the new DF develops under basal concentrations of progesterone into an ovulatory follicle. Conception rates from the timed-AI in this case would be near 0%. Thus, it is critical to control ovulation of a DF in response to the first GnRH not only to induce an accessory CL and increase circulating P4, but to control the age of the DF and control ovulation to the final GnRH-induced LH surge and avoid the asynchrony of ovulation as described. To ensure that cows respond to the first GnRH-induced LH surge of Ovsynch, cows must be on Days 6 or 7 of the estrous cycle [12]. Thus, it is absolutely imperative that cows be pre-synchronized before Ovsynch so that cows are on Days 6 or 7 of the estrous cycle at the first GnRH of Ovsynch.

The second treatment in the Ovsynch protocol, a luteolytic dose of PGF, is administered to induce luteolysis, thus enabling the DF of the new follicular wave to develop into a pre-ovulatory follicle. Our recent data indicate that lactating dairy cows only have a 77% chance of complete luteolysis before the final GnRH [35]. Even though cows have a high likelihood of responding to the final GnRH and ovulating, these data indicate that the chance of a pregnancy is

5% if circulating P4 at the time of the final GnRH is not less than 0.5 ng/mL. Also, when Ovsynch is initiated late in the estrous cycle, there is a high likelihood that the CL may undergo natural luteolysis before the PGF injection. If this happens, cows may have a natural estrus and ovulate early. If cows are not detected in estrus and inseminated accordingly, conception rates may be significantly reduced following timed-AI due to the asynchrony of AI and ovulation, i.e. timed-AI may occur well after ovulation.

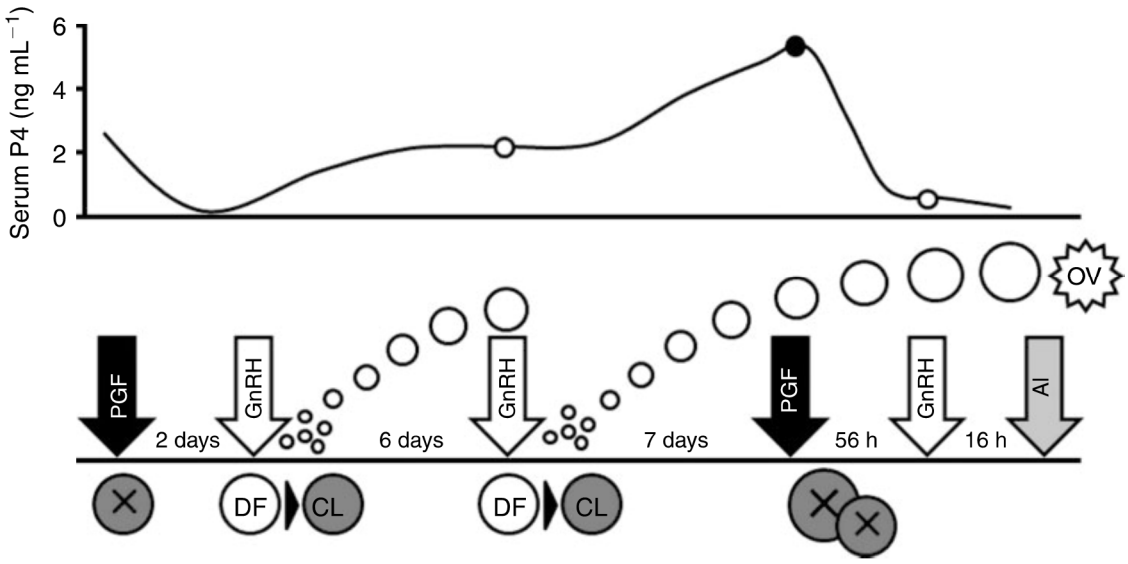

Figure 7.2. Description of dominant follicle (DF) and corpus luteum (CL) development and concentrations of progesterone in lactating dairy cows that responded (new CL and new follicular wave following GnRH or luteolysis following prostaglandin $F_{2\alpha}$ (PGF) to each treatment of a PGF (2 days) GnRH pre-synchronization scheme 6 days before the initiation of Ovsynch (GnRH, 7 days later PGF, 56 h later GnRH, 16 h later AI). x, luteolysis; OV, ovulation; AI, artificial insemination.

The third treatment in the Ovsynch protocol is 100mg of GnRH. This additional GnRH treatment is administered 48–60 h after PGF injection to induce a pre-ovulatory LH surge, trigger ovulation of the functional DF 24–32 h later [3] and release the oocyte to be fertilized following timed-AI. The chance of ovulation to this treatment is .95% even if luteolysis is not

complete before this injection [3]. As mentioned above, cows can have a synchronized ovulation but still not have a chance to become pregnant due to incomplete or prolonged luteolysis.

Evidence that P4 concentrations at time of PGF-induced luteolysis of Ovsynch are positively associated with fertility

Fonseca *et al.* [36] were the first to report that Holstein and Jersey dairy cows that became pregnant had greater concentrations of P4 in a 12-day period before AI compared with cows that did not become pregnant. In two studies conducted recently in our laboratory [6,35], P4 concentrations at the time of the PGF-induced luteolysis of Ovsynch had a substantial impact on the probability of a pregnancy in Holstein dairy cows (Figure 2.3). Thus, enhancing P4 concentrations before PGF-induced luteolysis is expected to enhance fertility. To induce an accessory CL following the first GnRH of Ovsynch, cows must be pre-synchronized (before Ovsynch) to ensure that cows are in a stage of the estrous cycle that has both a high probability of ovulation of a DF to the first GnRH and control of subsequent luteolysis with PGF, i.e. before endogenous luteolysis. In two previous studies [12,37] we tested and compared the rate of induction of accessory CL at Days 4, 5, 6, 7 or 8 of the estrous cycle at the time of the first GnRH of Ovsynch. The Day 6 interval (referred to as G6G) was found to result in a significantly greater percentage of cows ovulating to the first GnRH and inducing an accessory CL compared with the other intervals. In cows that responded to both PGF and GnRH pre-synchrony treatments and were on Day 6 of the cycle at time of first GnRH of Ovsynch, 97% developed an accessory CL, had significantly greater circulating P4 concentrations and a greater probability of a pregnancy [12].

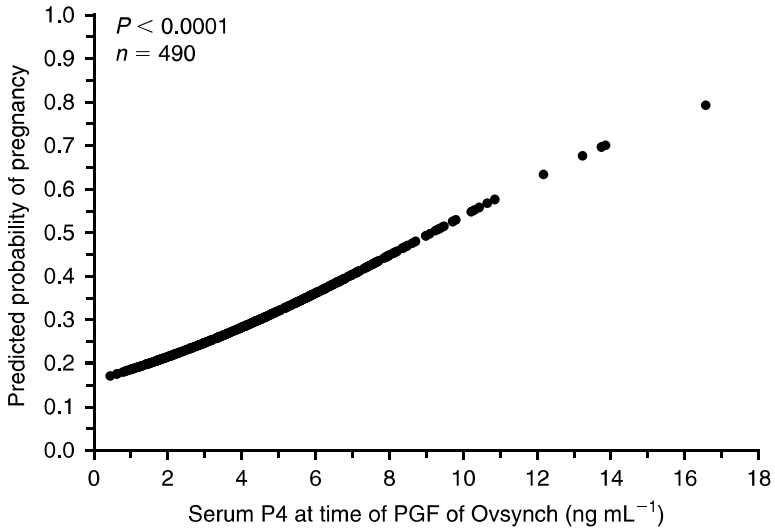

Figure 7.3. The predicted probability of pregnancy 39 days post artificial insemination based on circulating concentrations of progesterone (P4) at time of final prostaglandin $F_{2\alpha}$ (PGF) of Ovsynch in lactating dairy cows (n = 490) treated with pre-synchronization + Ovsynch and with functional corpus luteum (CL) at time of $PGF_{2\alpha}$ of Ovsynch.

Evidence that GnRH induction of accessory CL during Ovsynch increases P4 concentrations at time of PGF-induced luteolysis of Ovsynch

Multiple strategies have been tested previously to increase P4 before the PGF injection of Ovsynch. In studies that used exogenous P4 (progestin-releasing vaginal devices) to achieve greater concentrations of circulating P4 before AI, there was no significant increase in P4 on the day of PGF-induced luteolysis of Ovsynch or in resulting conception rates of cycling cows [38]. In cycling cows receiving progesterone-releasing vaginal devices, i.e. controlled internal drug release (Pfizer), concentrations of P4 were 2.7 versus 2.8 ng/mL in cows without a progesterone-releasing vaginal device [38]. Thus, use of a progesterone-releasing vaginal device does not represent a viable strategy to increase P4 concentrations and enhance fertility in lactating dairy cows. We therefore examined the possibility that GnRH inducement of an accessory CL during Ovsynch could be used to more effectively enhance P4 concentrations. As mentioned above, when cows were on Day 6 of the estrous cycle at the time of the first GnRH of Ovsynch, 97%

ovulated a first wave DF and formed an accessory CL. When the PGF of Ovsynch was administered 7 days later, cows that ovulated had both a primary Day 13 CL and a Day 7 accessory CL and correspondingly greater circulating concentrations of P4 at that time, compared with cows that did not receive GnRH and only had a Day 13 CL (Figure 2.4). Thus, presence of a young (Day 7) accessory CL during Ovsynch positively impacts P4 concentrations before AI.

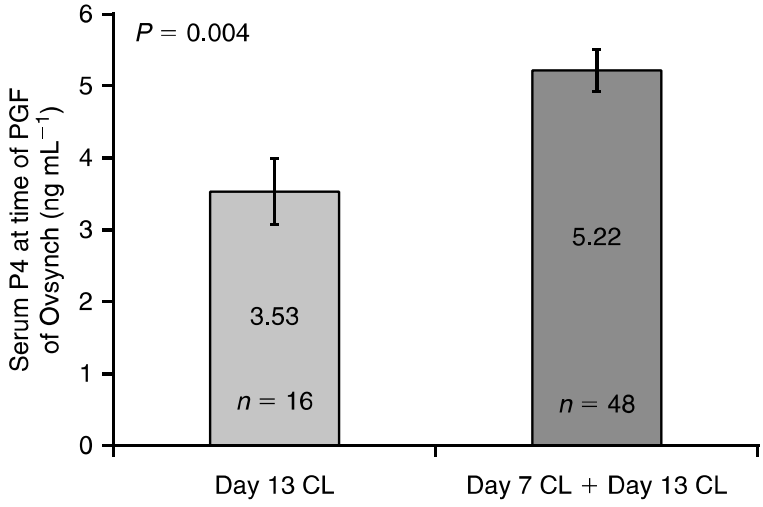

Figure 7.4. Average circulating concentrations of progesterone (P4) at prostaglandin $F_{2\alpha}$ (PGF) of Ovsynch in lactating dairy cows (n = 64) with a Day 13 corpus luteum (CL) versus a Day 13 CL with an additional Day 7 accessory CL.

SUMMARY

These data provide a basis for the concept that low progesterone in lactating dairy cows due to enhanced steroid metabolism may be the underlying cause of the low fertility that has plagued dairy herds for the past two decades. We have developed synchronization strategies that partially solve this problem. Enhancing the percentage of cows that respond to the first GnRH of Ovsynch allows for more cows with an accessory CL, greater concentrations of circulating progesterone at time of induced luteolysis and a greater chance of the establishment of pregnancy.

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CHAPTER 8

EFFECT OF INDUCING HIGH OR LOW SERUM CONCENTRATIONS OF PROGESTERONE DURING PRE- OR POST-DEVIATION OF THE OVULATORY FOLLICLE DEVELOPMENT ON DOUBLE OVULATION RATE AND FERTILITY OF LACTATING DAIRY COWS

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ABSTRACT

The objective of this experiment was to determine the effect of high vs. low progesterone during pre-dominance and/or dominance phase of ovulatory follicle development on follicular dynamics and fertility of lactating dairy cows. Progesterone (P4) was manipulated to reach high (H) or low (L) serum concentrations during the pre-dominance phase (days 0 to 4 of the wave) and dominance phase (days 5 to 7 of the wave) of a second follicular wave ovulatory follicle, creating 4 treatments: H/H, L/L, H/L, and L/H. Luteolysis was induced with $PGF_{2\alpha}$ on day 7 of the wave and ovulation was induced with GnRH 56 hours after $PGF_{2\alpha}$. Cows (n=558) received AI 16 hours following GnRH. Low P4 in either period increased double ovulation rate. Cows with double ovulations had greater pregnancies per AI (P/AI) on day 23 post-AI compared to cows with single ovulations. Cows with low P4 during the entire period of the ovulatory follicle development also had greater P/AI on day 23 post-AI compared to cows with high P4 during both phases. However, full term P/AI was not different between treatments. This was a result of the greater incidence of pregnancy losses between days 28 and 56 of gestation for cows with unilateral double ovulations compared to cows with single ovulation. Cows with single ovulation and low circulating P4 during the dominance period of follicle development also had increased pregnancy losses between days 28 and 56 of gestation compared to cows with single ovulation and high P4.

INTRODUCTION

Lactating dairy cows have significantly lower circulating concentrations of steroid hormones (progesterone and estradiol) than nulliparous heifers [1], which is associated with the increased metabolism of steroid hormones related to greater feed intake and milk production [2-6]. The decrease in concentrations of circulating steroid hormones is related with a series of

physiological changes that negatively impacts the fertility of lactating dairy cows [2]. Lactating dairy cows exhibited shorter duration of estrus [7], increased incidence of multiple ovulations [8] and larger ovulatory follicle and corpus luteum (CL) compared with dairy heifers [1].

During the last decade, fertility treatments (synchronization protocols) were developed to improve fertility of lactating dairy cows by controlling follicular and CL development, increasing circulating concentrations of steroids, synchronizing ovulation and increasing service rate (total animals inseminated during a period of time) [9-17]. However, the outcome of these fertility treatments remained highly compromised due to reduced concentrations of progesterone (P4) during the growth of the ovulatory follicle [18,19]. Several studies from our laboratory [9,18,20] and others [21-25] indicated the positive relationship between circulating P4 concentrations during the growth of the ovulatory follicle and fertility. A recent meta-analysis, including 25 randomized controlled studies with a total of 16,683 lactating dairy cows using the same fertility treatment, indicated that cows without a CL were benefited with supplementation of P4 during ovulatory follicular growth [22]. In addition, P4 supplementation tended to reduce the risk of pregnancy loss.

It has been demonstrated that fertilization rate is similar between cows with high and low circulating P4 during the development of the ovulatory follicle (~ 80%) [26]. However, cows with low circulating concentrations of P4 have lower conception rates on day 34 and 39 after artificial insemination (AI) than cows with high serum P4 [9,18,20,21,24,25]. Recent studies with superovulated lactating dairy cows and non-lactating beef cows indicated that high circulating P4 during superstimulation with FSH enhanced quality of day 7 embryos [27,28]. Furthermore, low circulating P4 during the growth of the ovulatory follicle in a fertility treatment was also related to an increase in pregnancy losses between days 29 and 60 post-AI in lactating

dairy cows [24]. These observations suggest that low serum P4 during the development of the ovulatory follicle might be one of the critical factors leading to the increase in early embryonic death in lactating dairy cows.

Moreover, oocytes from ovulatory follicles that developed under low progesterone concentrations but in prolonged dominance periods (persistent follicles) underwent early spontaneous germinal vesicle breakdown [29]. Cows with low serum concentrations of P4 have a high pulse frequency of luteinizing hormone (LH) due to a reduced negative feedback of P4 on pulses of LH [30]. Therefore, persistent follicles appear to be over-stimulated by a high pulse frequency of LH for an extended period of time. Fertilization rates of oocytes from persistent follicles are similar to the ones of oocytes from follicles that grew under greater serum P4 for a shorter dominance period [31]. However, early embryonic development appears to be compromised in cows with persistent follicles. Only 14% of the embryos derived from oocytes from persistent follicles developed normally to morula. On the other hand, 86% of embryos from oocytes from ovulatory follicles that developed under greater serum P4 and shorter dominance period developed to morula [31]. The effect of low circulating P4 concentrations on fertility of lactating dairy cows with synchronized follicular wave may be similar to the effects of the persistent follicle described. The ovulatory follicle that developed under low circulating concentrations of P4 may have been over-stimulated during the dominance period by a high pulse frequency of LH and might ovulate a less competent oocyte with future compromised development.

Although strong evidence indicates a role of insufficient levels of P4 during the growth of the ovulatory follicle in fertility of lactating dairy cows, there is still a lack of a controlled study determining causality between low levels of serum P4 and impaired fertility of lactating dairy

cows. Clarification is also needed regarding the mechanisms by which low circulating concentrations of P4 during the ovulatory follicle development limit the improvements of fertility treatments in lactating dairy cows. Thus, the main objective of this study is to determine the effects of low serum concentrations of P4 during different periods of ovulatory follicle development (pre-deviation, post-deviation, or both) on fertility of lactating dairy cows. Our central hypothesis is that reduced serum concentrations of P4 during the dominance period of the ovulatory follicle have the potential to decrease pregnancy success after artificial insemination and increase pregnancy loss during gestation.

MATERIALS AND METHODS

Cows, housing and materials

This experiment was conducted on a commercial dairy farm (Nobis Dairy Farm, St. Johns, MI) with approximately 1,000 lactating dairy cows that were milked 3 times daily. Average herd milk production during this period was approximately 42 kg/cow/day. Cows were housed in free stall barns, fed a TMR once daily, and had free access to food and water. The TMR consisted of corn and alfalfa silages and corn-soybean meal-based concentrates formulated to meet or exceed nutrient recommendations for lactating dairy cows (NRC, 2001).

All injections in this experiment were administered with single-dose syringes with 3.5 cm needles in semimembranosus or semitendinosus muscles of cows by trained personnel from our laboratory. All $PGF_{2\alpha}$ treatments in this experiment used 25 mg of dinoprost (5 mL of Lytalyse, Zoetis, Kalamazoo, MI). All GnRH treatments used 100 μg of gonadorelin (2 mL of Factrel, Zoetis, Kalamazoo, MI) unless otherwise stated. Vaginal P4 controlled internal drug release devices (CIDR; 1.38 g of P4, Eazi-Breed™ CIDR® Cattle Insert; Zoetis) utilized were new or

used. Used CIDRs were removed from lactating dairy cows after being inserted once for 3 or 4 days, washed with soap and water and disinfected with chlorhexidine gluconate solution (0.03%) for 2 hours as previously described by Zuluaga and Williams [32]. Used CIDRs were used in the present experiment to keep sub-luteal serum concentrations of P4. All animal procedures described in this manuscript were approved by The Institutional Animal Care and Use Committee at Michigan State University.

Experimental design

Pre-treatment

Weekly cohorts of lactating dairy cows (n = 1,051) between 57 to 63 days in milk (first service) and cows diagnosed not pregnant 39 days after previous AI (second or greater services) were pre-synchronized with corresponded to an injection of $PGF_{2\alpha}$ and an injection of GnRH 2 day later (G7G [33]) to induce a new estrous cycle. Ovaries were scanned and mapped on day of GnRH by transrectal ultrasonography as previously described by Martins et al. [34] using a color Doppler MyLabOne ultrasound with a 6-10 MHz multi-frequency linear array probe (Esaote, Indianapolis, IN). Height and width of the largest cross-section size of follicles with antrum average diameter > 7 mm and CL were measured using build-in calipers. Corpus luteum with a fluid-filled central cavity also had the large cross-section of the cavity measured. Smaller follicles (≤ 7 mm) had their average diameter estimated based on on-screen lateral grids. All follicles and CL were draw and had their measurements recorded in an ovarian map for each cow with date of examination. Ovaries were scanned with ultrasound again 2 days following the GnRH of the pre-synchronization to determine ovulation and CL regression. Ovulation was characterized by the disappearance of a follicle(s), followed by detection of a newly formed CL

on the same site of the ovary, where the dominant follicle (DF) was present. Corpus luteum regression was determined by a decrease in the maximum luteal size and disappearance of luteal blood flow area using the color function of the ultrasound machine as described previously [35,36]. Luteal regression was reevaluated 5 days later on the next ultrasound examination of the ovaries. Cows with CL regression and ovulation after the presynchronization were determined to have started a new cycle on day of GnRH (day 0 of the new estrous cycle).

Only cows that started a new cycle were blocked by parity and AI service number then randomly assigned to four treatments initiated on day 7 of the estrous cycle. On this day, a second GnRH (200μg of gonadorelin, 4 mL of Factrel, Zoetis, Kalamazoo, MI) injection was administered to induce a second follicular wave; which was the ovulatory follicular wave. Ovulation was determined as previously described, and only cows that ovulated to this GnRH and started a second follicular wave on day 7 of the cycle were used in this experiment. Day of GnRH was considered day 0 of treatment and of the synchronized ovulatory follicular wave. All cows considered in the experiment started the ovulatory follicular wave at the same day and had similar ovulatory follicle antral age detected by ultrasonography examination.

Treatment

During treatment period, serum P4 was manipulated to reach high (H) or low (L) concentrations during the pre-dominance phase (days 0 to 4 of the wave) and dominance phase (days 5 to 7 of the wave) creating 4 groups: High/High (H/H), Low/Low (L/L), Low/High (L/H), and High/Low (H/L) as illustrated in Figure 3.1. Day 4 of the ovulatory follicle wave was defined as the day of DF deviation based on previous studies [37]. A combination of $PGF_{2\alpha}$ and used CIDR was applied to create low serum P4. New CIDR (1 or 2 devices) in combination with

a CL (original and/or accessory) was used to produce a high serum P4.

Cows in the H/H treatment received a new CIDR on treatment day 1 that was replaced for a new one on day 4. Cows in the H/L treatment also received a new CIDR on treatment day 1. This CIDR was replaced by a used CIDR on day 4, and an injection of $PGF_{2\alpha}$ was administered. Another injection of $PGF_{2\alpha}$ was administered on day 5. Cows in the L/H treatment received an injection of $PGF_{2\alpha}$ on treatment day 0, and a used CIDR was inserted on day 2. This CIDR was replaced for 2 new CIDRs on day 4. One was removed on day 7 and the other on day 8. Cows in the L/L treatment received an injection of $PGF_{2\alpha}$ on day 0 and a used CIDR on day 2, which was replaced for another used CIDR on day 4 followed by a $PGF_{2\alpha}$. A third $PGF_{2\alpha}$ was administered on day 5.

All cows of the experiment received $PGF_{2\alpha}$ on day 7 to induce luteolysis. On day 8, all CIDRs were removed and, a second $PGF_{2\alpha}$ injection was administered to ensure complete luteal regression. Approximately 32 hours later, the last injection of GnRH was administered to synchronize ovulation of the ovulatory follicle. Ovulation was determined 2 days following this injection as previously described, and only animals that ovulated were included in the analysis. Thus, all cows (N = 562) in the study had similar ovulatory follicle age at time of GnRH induced-LH surge on day 9 and by ovulation between day 10 and 11. Corpus luteum regression after final PGF was determined by a decrease of the maximum luteal size and disappearance of luteal blood flow area using the color function of the ultrasound machine on treatment days 9 and 11 as determined to the first PGF of pre-treatment. All cows were considered to have complete induced luteolysis on treatment day 9. Final number of cows for each treatment was: H/H, n =134; H/L, n = 137; L/H, n = 152; and L/L, n =139.

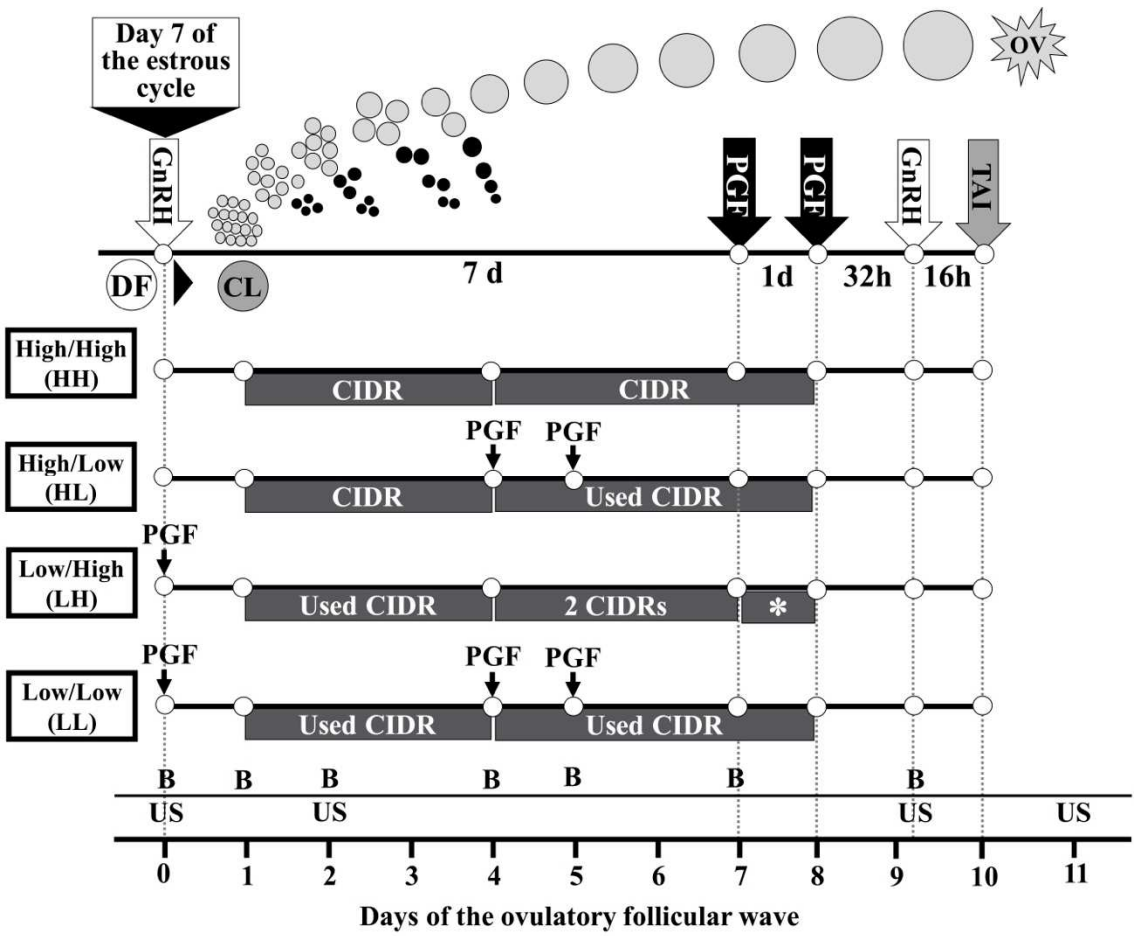

Figure 8.1. Schematic illustration of experimental design with time of hormone injections (PGF and GnRH), blood collection (B) and ultrasonography examination of ovaries (US) for each treatment (H/H, H/L, L/H and L/L) in lactating dairy cows. PGF= $PGF_{2\alpha}$; TAI = timed-artificial insemination; DF= dominant follicle; CL= corpus luteum; OV= ovulation; CIDR= Intra-vaginal progesterone controlled internal drug release device; Used CIDR= CIDR used by lactating dairy cows for 3 or 4 days once, washed with soap and water and disinfected with chlorhexidine gluconate solution (0.03%) for 2 h. * One CIDR was removed on d 7, and the other CIDR removed on d 8.

Blood collection and hormone assays

Blood samples were collected from the coccygeal vein or artery of a subset of cows on treatment days 0, 1, 2, 4, 5, 7, and 9 (n = 308) using Vacutainer tubes without anticoagulant (BD Vacutainer, Franklin Lakes, NJ) to measure serum concentrations of P4. Additional blood

samples were collected on days 7, 14, 16, 20, 23 and 28 after AI to also assess serum levels of P4 in the majority of cows of the experiment (n =544). Serum concentrations of Pregnancy-Specific Protein B (PSPB) were assess days 16, 20, 23 and 28 post-AI (n = 544). Following collection, samples were refrigerated, transported to our laboratory and maintained at 4 °C overnight. Serum was separated within 24 hours after collection by centrifugation at 2000 x g for 20 min at 4 °C and stored at -20 °C for later P4 analyses.

Serum concentrations of P4 were measured using a commercially available solid-phase RIA kit (DIAsource ImmunoAssays, Louvain-la-Neuve, Belgium). Each assay contained all samples from the same cow arranged in sequence and with single samples. Number of treatments per assay were homogeneous and arranged randomly. Intra- and inter-assay coefficient of variation (CV) were 21.8% and 24.7%, respectively. Serum concentrations of PSPB were measured using a commercially available quantitative sandwich ELISA assay kit (BioPRYN; BioTracking LLC, Moscow, ID, USA), which was developed based on data from Sasser et. al (1986). Each 96-well test plate was run with two replicate of each standard (0.125, 0.25, 0.5, 1, 2, and 4 ng/ml), control level (low and high) and samples. All samples from the same cow on days 16, 20, 23 and 28 were run in the same plate. In addition, each plate contained the most uniform number of treatments per plate arranged randomly. A linear least square regression was used to calculate the standard curve of each plate, which was used to determine the concentration of PSPB in each sample of the plate. Value of each serum sample was calculated by the average of the two replicate values. The CV of each sample was also determined, and samples with CV > 10% had their PSPB measured in a new assay. Inter- and intra- assay was determined for each control level. Intra- and inter-assay CV were 3.9 and 5.8 %, respectively.

Pregnancy diagnosis and calving records

Pregnancy was determined on day 23, 28, 35, 56, 117 ± 7 and 194 ± 7 post-AI. Pregnancy diagnosis on day 23 and 28 post-AI were determined by serum concentrations of PSPB. The predictor for pregnancy diagnosis on day 28 post-AI were determined by receiver operating characteristic (ROC) curve analysis using the MedCalc software package (MedCalc Software, Oostende, Beligium) and pregnancy diagnosis at 35 day post-AI by ultrasonography examination as the true positive. The cut-off point for serum PSPB at 28 day post-AI in our study was 0.60 ng/mL, which had sensitivity of 100% and specificity of 91.6%. Cows with serum PSPB at 28 days after AI > 0.60 ng/mL were considered pregnant at that time.

To identify the best predictor for pregnancy diagnosis on day 23 post-AI, a ROC curve analysis was performed with pregnancy diagnosis at day 28 post-AI set as the true positive. Two variables were tested: serum PSPB concentration on day 23 post-AI and percentage increase in serum PSPB concentration from basal to day 23 post-AI. Basal concentration of PSPB was calculated using the average of PSPB on days 16 and 20 post-AI. Youden index (*J*) for both variables were calculated and the maximum value of the index was used to stipulate the optimum cut-off point for each variable. Percentage increase in serum concentrations of PSPB from basal to day 23 post-AI was identified as a more accurate predictor with 98.0 % sensitivity and 97.0 % specificity compared to serum PSPB on day 23 post-AI that had 92.8 % sensitivity and 97.0 % specificity. Cows with an increase in serum PSPB from basal to day 23 after AI of more than 28.4 % were considered pregnant at that time. Only six cows were false negatives; however, they were also considered to be pregnant on day 23 since they were identified as pregnant on day 28 post-AI. Other six cows with missing samples on day 23 post-AI but with positive pregnancy diagnosis on day 28 post-AI were also considered pregnant on day 23 post-AI.

Pregnancy diagnoses on day 35 and 56 after AI were performed by farm veterinarians blind to treatment using an Ibex Pro ultrasound (E.I. Medical Imaging, Loveland, CO). Pregnancy was confirmed by embryo presence with heartbeat. Pregnancy diagnosis on day 117 ± 7 and 194 ± 7 post-AI were determined by milk concentrations of pregnancy-associated glicoproteins (PAGs) as a farm management routine. Milk samples were collected on test days by technicians from NorthStar Cooperative (Lansing, MI). Samples were transported to a commercial laboratory in Lansing, MI on the same day (AntelBio Laboratory) and were analyzed by a commercial ELISA kit according to manufacture instructions (IDEXX Laboratories Inc., Westbrook, ME). In a case of a negative pregnancy diagnosis by milk sample, farm veterinarians reconfirm the test result by transrectal palpation. Pregnancy diagnosis on day 35, 56, 117 ± 7 and 194 ± 7 post-AI and calving information of each cow for the present study was retrieved from the dairy herd management computer software (PCDart, DRMS, Releigh, NC). Calving records retrieved included date of calving, twin, gender and stillborn.

Statistical analyses

Binomial variables were analyzed using logistic regression with a generalized linear mixed model implemented with the GLIMMIX procedure of SAS (Version 9.4, SAS Inst., Inc., Cary, NC). The model considered treatments (H/H, H/L, L/H and L/L), parity category (primiparous or multiparous) and service category (first AI or second and greater AI) as fixed effects. Two-way interaction of treatment and parity or service category was only considered in the model if $P <$ 0.20. Continuous variables were analyzed using a linear mixed model applying the MIIXED procedure of SAS with the same fixed effects and random effect as describe for the GLIMMIX procedure. Treatment and parity number interaction was only considered in the model if $P <$

0.20. Normal distribution of the residuals was tested with studentized residual plots for each variable. Residuals were considered normally distributed for all variables. Differences in the percentage of cows with ovulations on left vs. right ovary vs. bilateral were analyzed by chi-square test using the FREQ procedure of SAS. All analysis used a two-tailed test and probabilities values were considered different when ≤ 0.05 and a trend for difference when ≤ 0.10.

Treatment effects were not confounded by parity and service number, since parity ($P = 0.97$) and service ($P = 0.97$) number were balanced between treatments. Mean ± SEM parity number for all cows was 2.3 ± 0.1, ranging from 1 to 8. Service number mean ± SEM was 1.8 ± 0.1, ranging from 1 to 8.

Analyses of luteal function following AI

Cows were considered to undergone complete luteolysis following AI when serum concentrations of P4 were < 2.21 ng/mL. This threshold was calculated by the mean serum concentration of P4 at treatment day 9 (1.17 ng/mL) plus 2 times the standard deviation (0.52 ng/mL). Serum P4 at treatment day 9 was used as a parameter since all cows were considered to have complete luteolysis at this time.

RESULTS

Effect of treatment on circulating concentrations of P4 during the pre-ovulatory follicle development, pre-ovulatory follicle diameter and double ovulation rate following treatment

Figure 3.2 shows that treatments induced high (H) or low (L) circulating concentrations of P4 during the first 4 days of the ovulatory follicle development (pre-dominance phase) or during the last 3 days prior to PGF-induced luteolysis (dominance phase) as intended by the

experimental design. Cows treated with L/H and L/L had lower serum P4 compared to cows treated with H/L and H/H during the pre-dominance phase of the ovulatory follicle development. Moreover, cows treated with H/L and L/L had lower serum P4 compared to cows treated with L/H and H/H during the dominance phase of the ovulatory follicle development. Cows treated with H/H had greater serum P4 compared to cows treated with L/H during the dominance phase, indicating that treatment with two new P4 vaginal implants (CIDR) and the presence of an accessory CL formed on day 1 of treatment were insufficient to reach similar serum P4 levels compared to cows with 2 CLs (mature and accessory CLs) treated with one new CIDR.

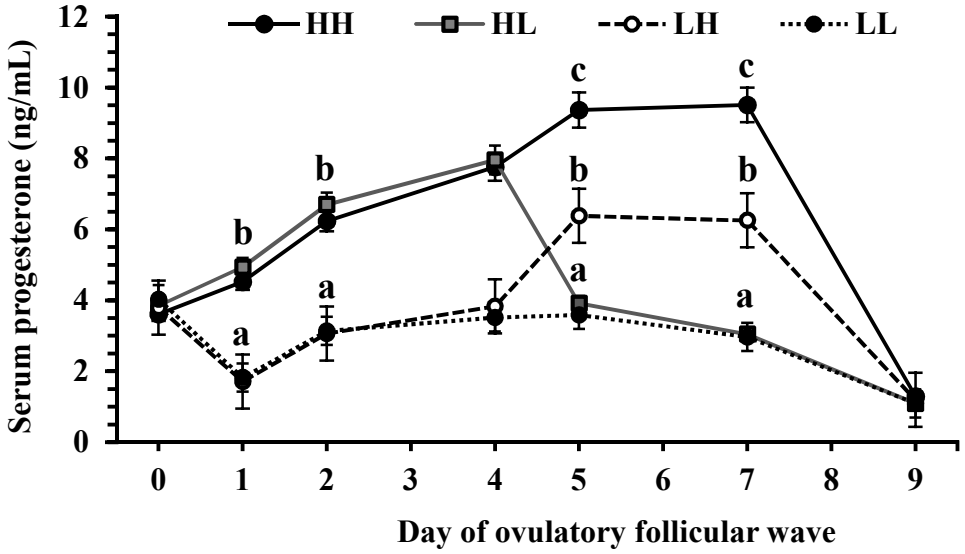

Figure 8.2. Serum concentrations of progesterone for lactating dairy cows (n = 308) during treatment period. Data are shown as mean ± SEM. Different letters (a, b, c) within a day represent differences between least square means ($P < 0.05$).

Treatment differences found in serum levels of P4 were further validated by the results of pre-ovulatory follicle diameter presented in Figure 3.3. Induction of low serum P4 during both stages of follicle development increased pre-ovulatory follicle diameter. However, a greater effect of low serum P4 was observed during the period of dominance. In cows with single ovulation, the effect of low P4 during both periods of follicle development (L/L) was even

greater compared to low P4 during only one stage of development (L/H or H/L). Cows with single ovulation had greater ($P < 0.001$) pre-ovulatory follicle diameter compared to cows with double ovulation.

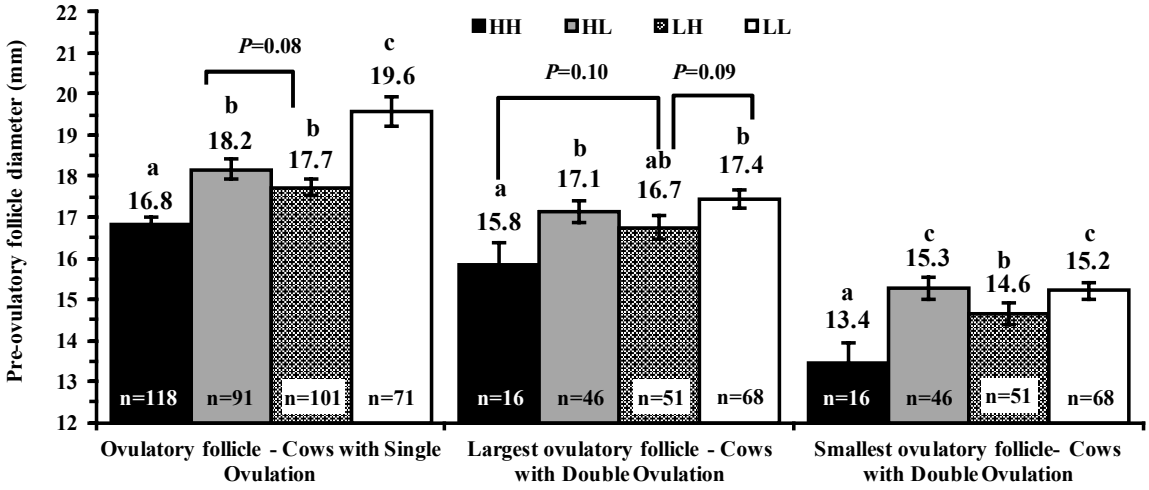

Figure 8.3. Effect of treatment on pre-ovulatory follicle diameter for cows with single or double ovulation. Data are shown as mean ± SEM. Different superscript within category of pre-ovulatory follicle (single ovulation or larger follicle in double ovulation, or smaller follicle in double ovulation) represent differences (a,b,c; $P < 0.05$) or a tendency for differences (A,B; $P \leq 0.10$) between least square means.

Induction of low P4 during follicle development also increased double ovulation rate following treatment (Figure 3.4A). Cows treated with H/L and L/H had similar double ovulation rate following treatment and greater rate compared to cows in H/H. Additionally, a greater effect of low P4 on double ovulation rate was observed when low P4 was induced during the entire follicle development (L/L). Double ovulation occurrence during pre-treatment was also related to double ovulation rate following treatment indicating that other individual factors besides hormonal environment also play a role on double ovulation incidence in lactating dairy cows (Figure 3B). As frequency of double ovulation during pre-treatment increased the probability of double ovulation following treatment was greater (Figure 3.4B). Interaction between treatment

and double ovulation occurrence during pre-treatment was not observed ($P < 0.95$) demonstrating that the same treatment had similar effect across different profiles of double ovulatory response during pre-treatment (Figure 3.5).

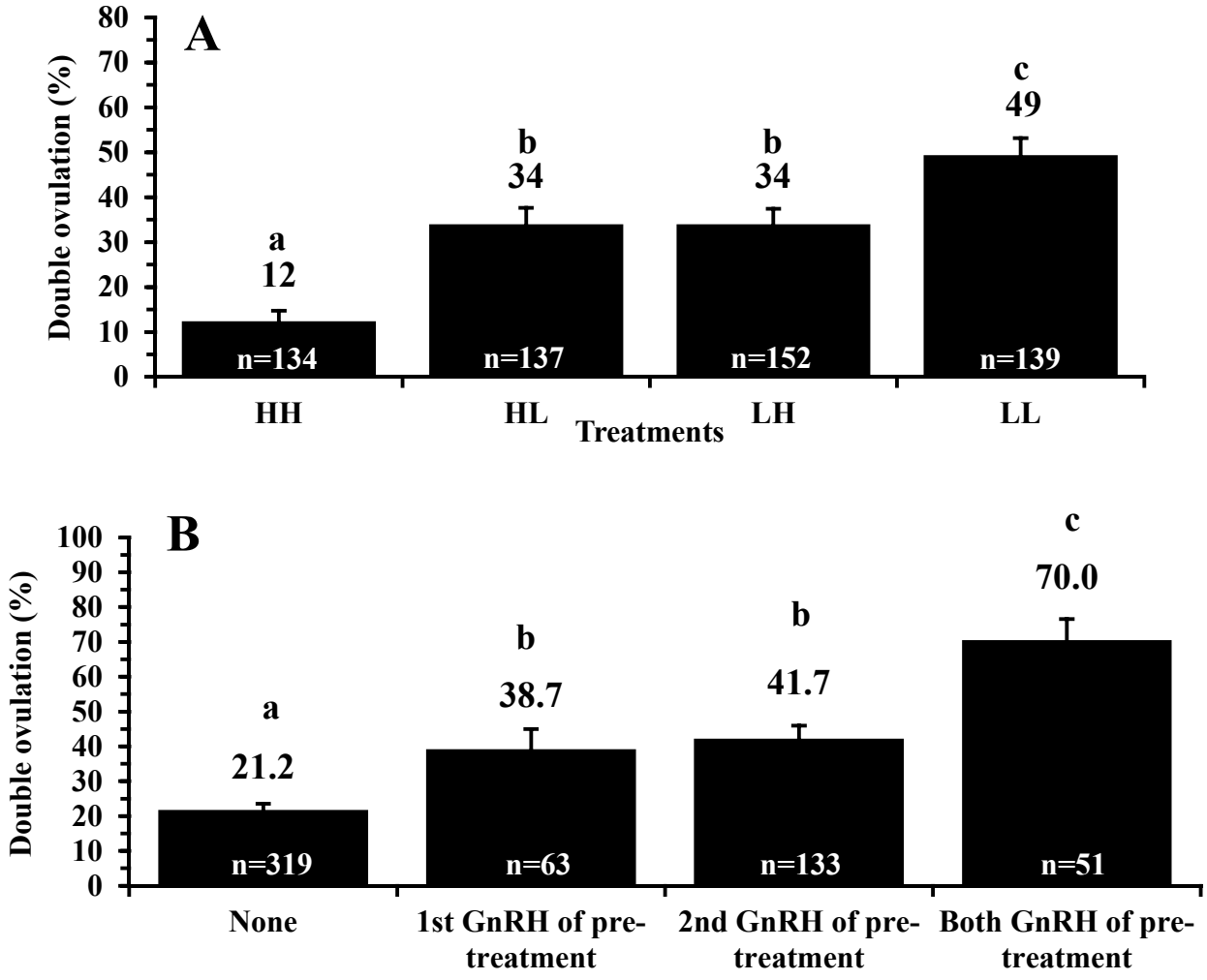

Figure 8.4. Effect of treatment (**A**) and previous double ovulation occurrence during pre-treatment (**B**) on double ovulation response for the last GnRH of treatment. Data are shown as mean ± SEM. Different letters (a, b, c) represent differences of least square means ($P < 0.05$).

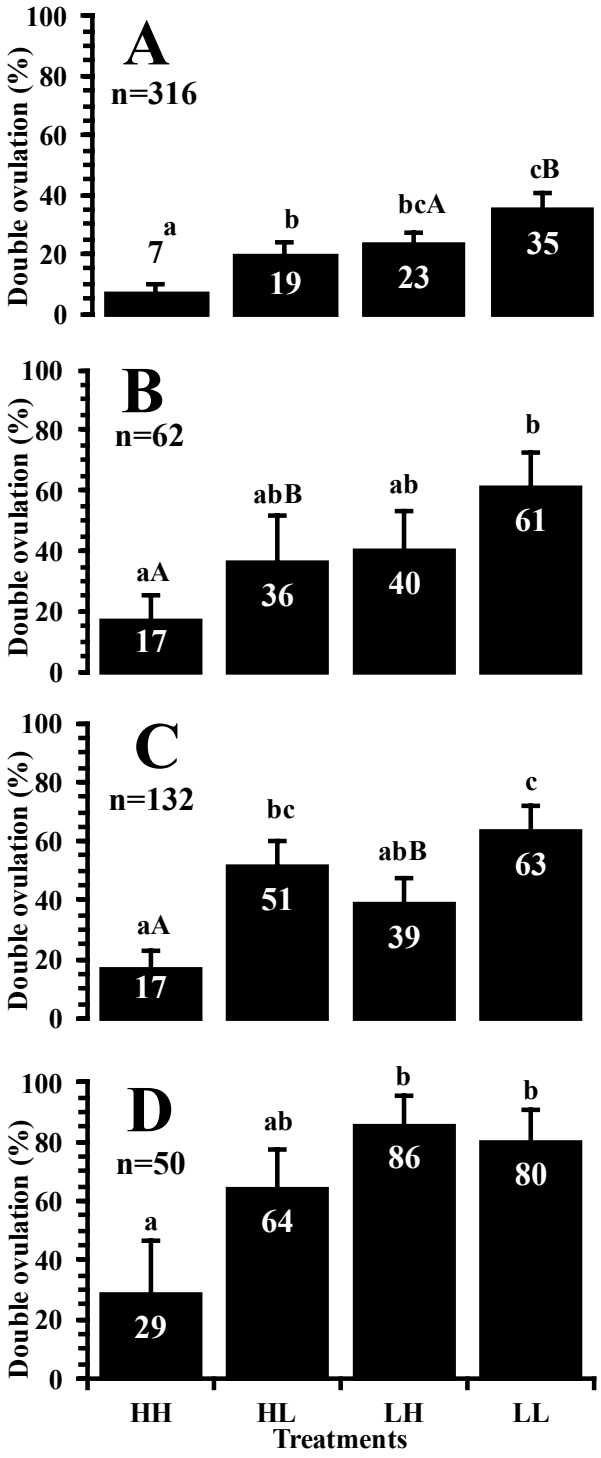

Figure 8.5. Effect of treatment on double ovulation rate for cows with (A) no double ovulation during pre-treatment, (B) double ovulation after 1st GnRH of the pre-treatment, (C) double ovulation after 2nd GnRH of the pre-treatment, and (D) double ovulation of both GnRH of the pre-treatment. Data are shown as mean ± SEM. Different superscript represent: differences (a, b, c; $P < 0.05$) or tendency for difference (AB; $P < 0.10$). between least square means .

Treatment did not affect ovulation side for cows with single or double ovulation (Table 3.1). A greater percentage of cows with single ovulation had ovulatory response in the right ovary compared to the left ovary. Cows with unilateral double ovulation had a tendency to have greater ovulatory response in the right ovary compared to the left ovary.

Table 8.1. Percentage of cows with ovulations on left and/or right ovary within treatments.

	Single ovulation			Double Ovulation[1]				
				Unilateral		Bilateral[1]		
Treatments	**Left ovary**	**Right ovary**	***P***	**Left ovary**	**Right ovary**	**Both ovaries**	***P****	***P* for unilateral left vs. right double ovulation**
HH, % (n/n)	35.6 (42/118)	64.4(76/118)	0.002	25.0 (4/16)	25.0 (4/16)	50.0 (8/16)	1	1
HL, % (n/n)	42.9 (39/91)	57.1 (52/91)	0.17	21.7 (10/46)	28.3 (13/46)	50.0 (23/46)	0.82	0.53
LH, % (n/n)	37.6 (38/101)	62.4 63/101)	0.01	21.6 (11/51)	29.4 (15/51)	49.0 (25/51)	0.72	0.43
LL, % (n/n)	39.4 (28/71)	60.6 (43/71)	0.08	16.4 (11/67)	32.8 (22/67)	50.8 (34/67)	0.16	0.06
Treatment differences (*P*)	0.75				0.98			
Total, % (n/n)	38.6 (147/381)	61.4 (234/381)	<0.001	20.0 (20/180)	30.0 (54/180)	50.0 (90/180)	0.17	0.06

[1]Three cows had triple ovulation with double ovulation in one ovary and single ovulation in the other ovary. These cows were considered to have bilateral ovulation.

*Chi-square tested if proportions differ from the following: 25 % unilateral double ovulation in the left ovary, 25 % unilateral double ovulation in the right ovary and 50 % bilateral double ovulation.

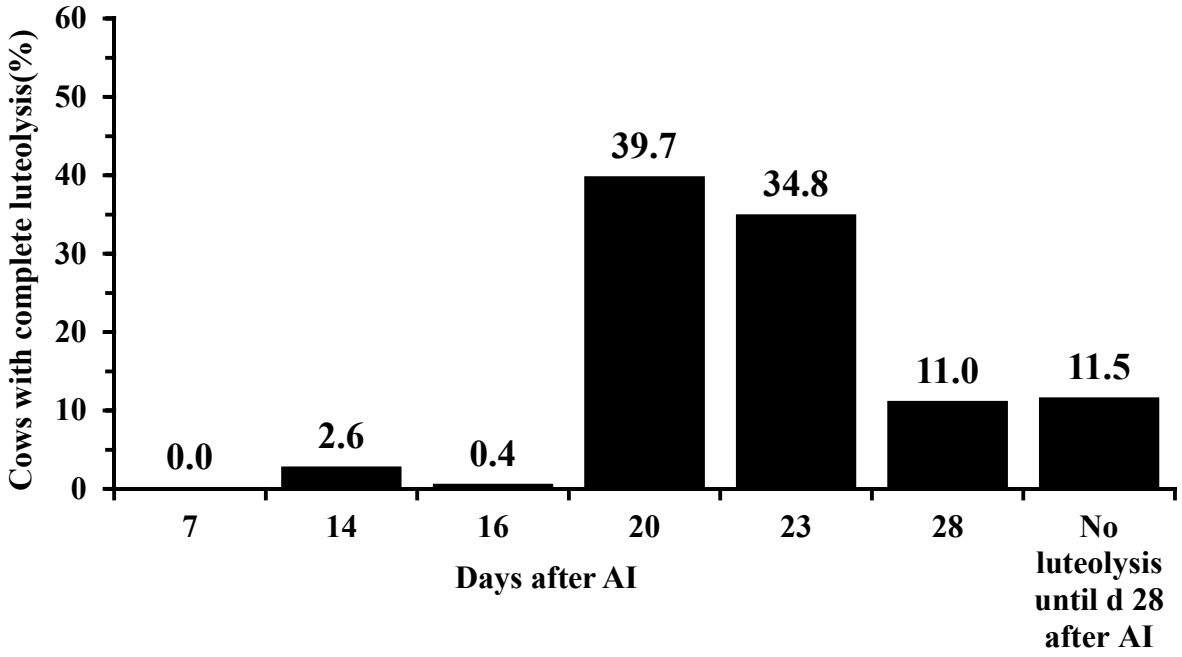

Figure 8.6. Percentage of cows not pregnant on day 23 after AI that were detected with complete luteolysis on days 7, 14, 16, 20, 23 and 28 following AI or not detected with complete luteolysis on any of these days after AI.

Analyses of luteal function and serum concentrations of PSPB following treatment

There was no effect of treatment (P = 0.38) or double ovulation following treatment (P = 0.81) on distribution of cows detected with luteolysis across different days after AI (Figure 3.6). The majority of non-pregnant cows on day 23 post-AI had complete luteolysis detected on day 20 or 23 after AI. A very low percentage (3.0 %) of non-pregnant cows on day 23 post-AI were considered to undergo complete luteolysis on or prior day 16 following AI (Figure 5). These results indicated that cows were synchronized following treatment and that treatments did not enhance percentage of cows with short luteal phase. About 11.5 % of non-pregnant cows on day 23 post AI were considered to not have complete luteolysis by day 28 after AI. Neither treatment (P = 0.20) nor double ovulation (P = 0.44) influenced the percentage of non-pregnant cows on day 23 post-AI without luteal regression by day 28 post AI.

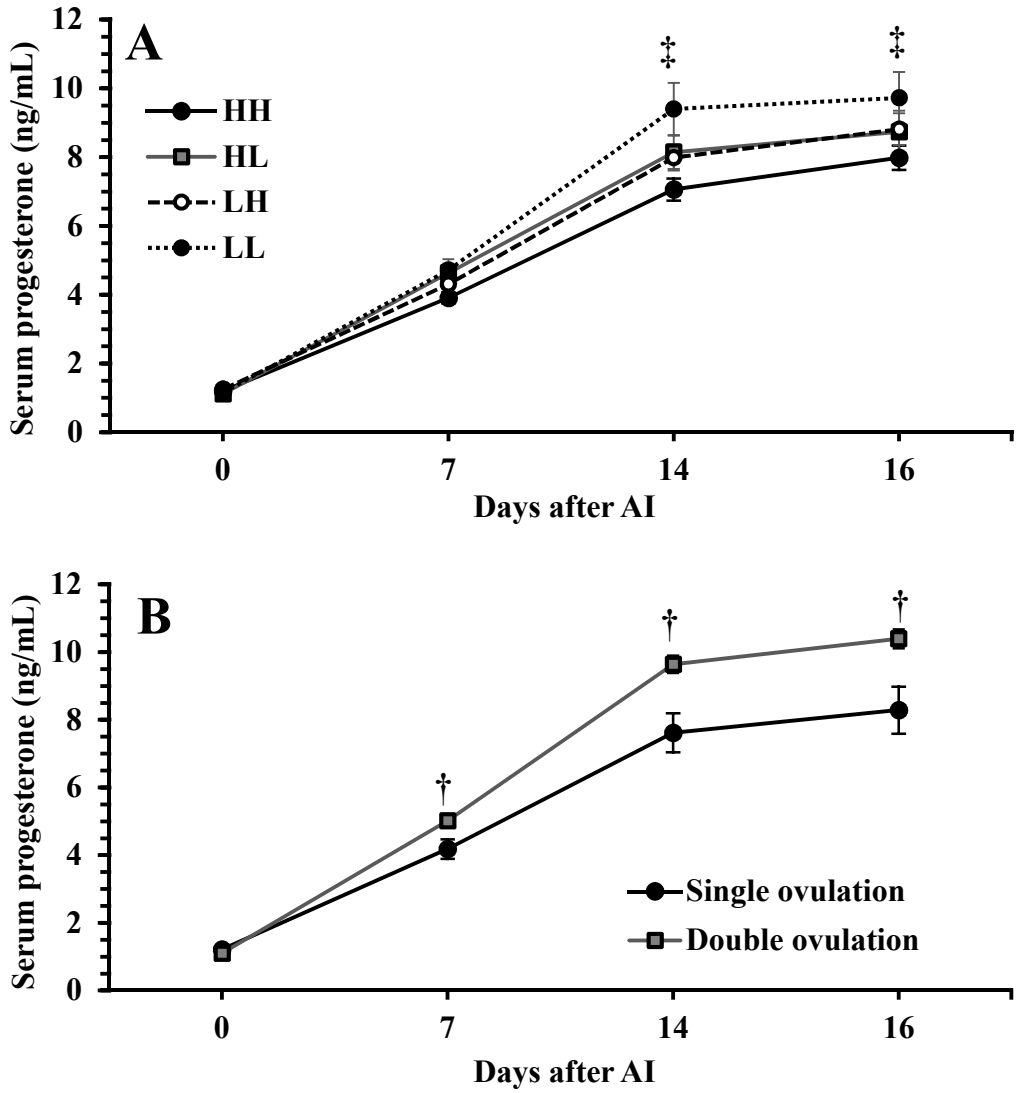

Figure 8.7. Mean ±SEM serum concentrations progesterone for cows without luteolysis until day 16 after AI according to (A) treatments or (B) type of ovulatory response after the last GnRH of treatment (single ovulation, unilateral double ovulation or bilateral double ovulation). Pairwise differences ($P < 0.05$) within a day are represented as follows: ‡LL vs. HH in (A); † single ovulation vs. double.in (B).

Treatment had an effect on serum concentrations of P4 following AI (Figure 3.7A). When only considering cows that did not undergone luteolysis until day 16 post-AI, cows treated with L/L had greater serum P4 on day 14 and 16 after AI compared to cows treated with H/H. This difference seems to be related to the greater percentage of cows with double ovulation in L/L treatment compared to H/H since cows with double ovulation also had greater serum P4 on

day 7, 14 and 16 after AI (Figure 3.7B). Considering only cows pregnant on day 28 post-AI, L/L cows also had greater serum P4 on day 16 post-AI compared to the other treatments (Figure 3.8A). Cows diagnosed pregnant with ultrasonography examination at day 35 post-AI had increased serum PSPB on day 23 and 28 post-AI compared to non-pregnant cows (Figure 3.9).

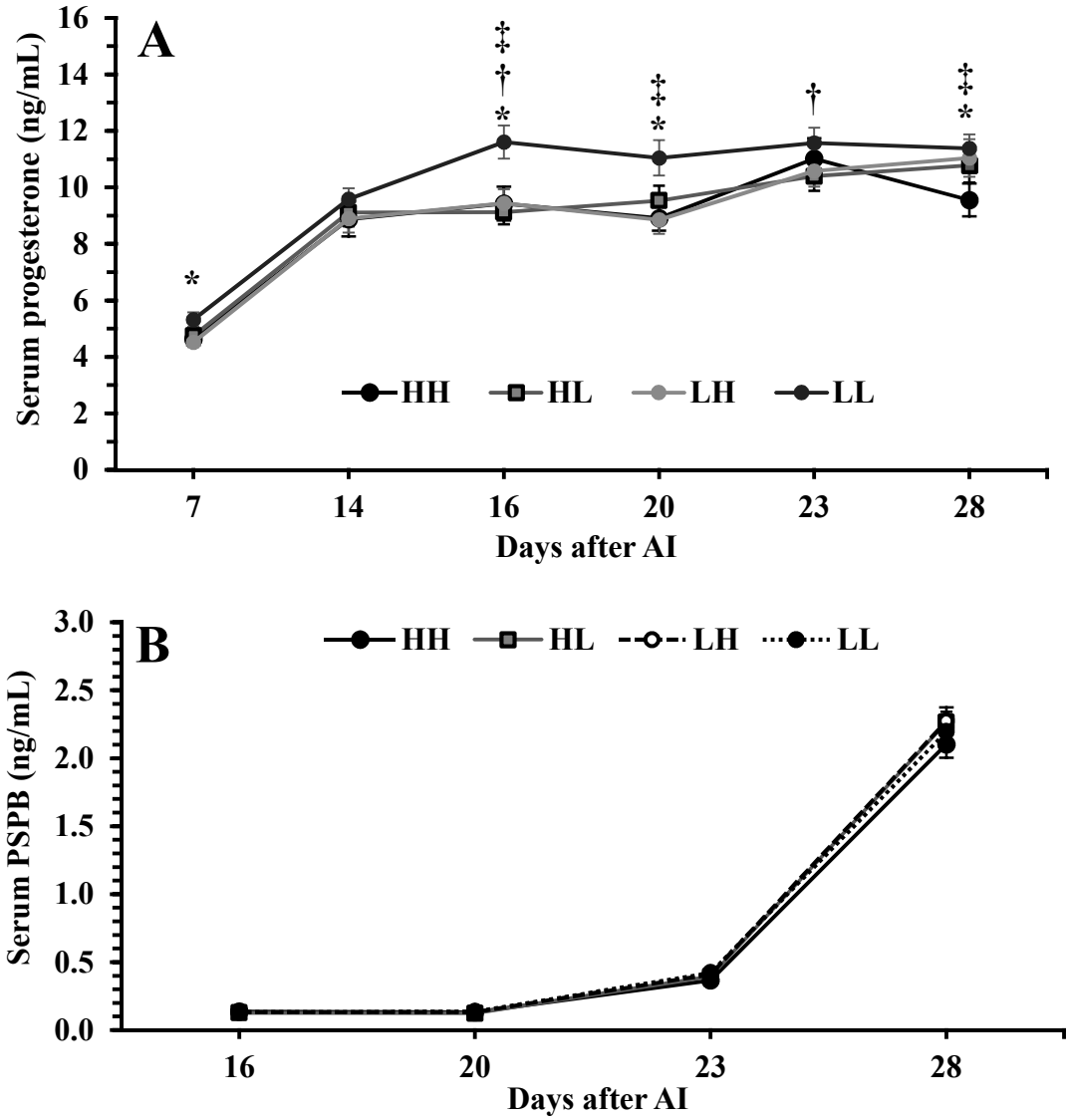

Figure 8.8. Mean ±SEM serum concentrations of PSPB (A) and progesterone (B) for cows pregnant at day 28 after AI according to treatments. Pairwise differences ($P < 0.05$) within a day are represented as follows: *LL vs. LH; † LL vs. HL; ‡LL vs. HH.

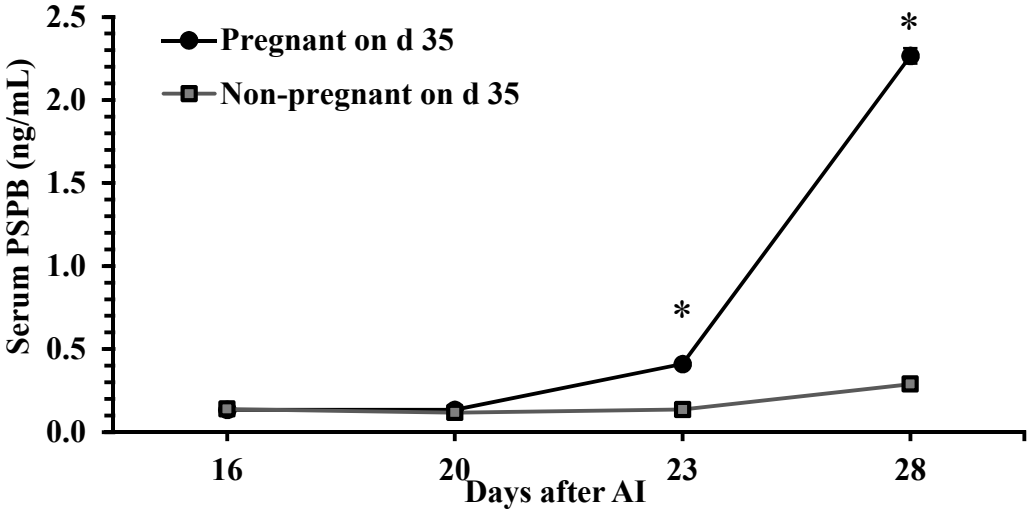

Figure 8.9. Mean ±SEM serum concentrations of PSPB for cows pregnant and non-pregnant on day 35 post-AI. * Asterisk represents differences of least square means within a day ($P < 0.001$).

The increase in serum concentrations of PSPB from basal (average serum concentration of PSPB on day 16 and 20) to day 23 post-AI was indicated as an accurate marker for pregnancy diagnosis on day 23 after AI in the present study. Treatment did not influence serum concentrations of PSPB on day 23 and 28 post-AI (Figure 3.8A) for cows detected pregnant on day 28 after AI. However, cows with bilateral double ovulation had significantly increased serum PSPB days 23 and 28 post-AI compared to cows with single ovulation or cows with unilateral double ovulation (Figure 3.10B). Cows pregnant on day 28 with double ovulation also had greater serum P4 compared to cows with single ovulation (Figure 3.10A). Cows with unilateral double ovulation had similar serum P4 compared to cows with bilateral double ovulation on days 7, 16, 20, 23 and 28 post-AI (Figure 10A). Day 14 was the only time point that cows with unilateral double ovulation had greater serum P4 compared to cows with bilateral double ovulation (Figure 3.10A).

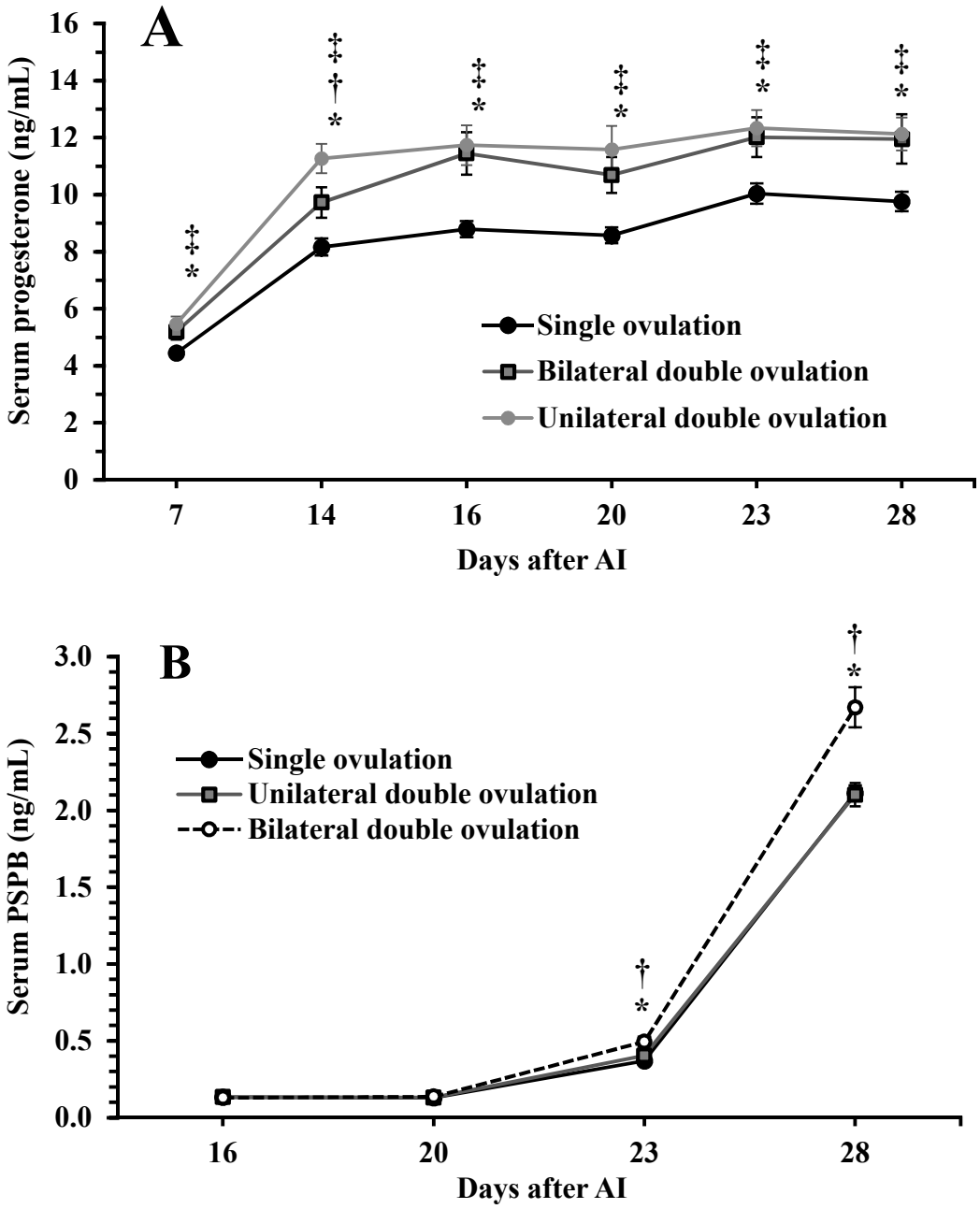

Figure 8.10. Mean ±SEM serum concentrations of PSPB (A) and progesterone (B) for cows pregnant at day 28 after AI with single ovulation and unilateral and bilateral double ovulation. Pairwise differences ($P < 0.05$) within a day are represented as follows: *Single ovulation vs. bilateral double ovulation; † unilateral double ovulation vs. bilateral double ovulation; ‡single ovulation vs. unilateral double ovulation.

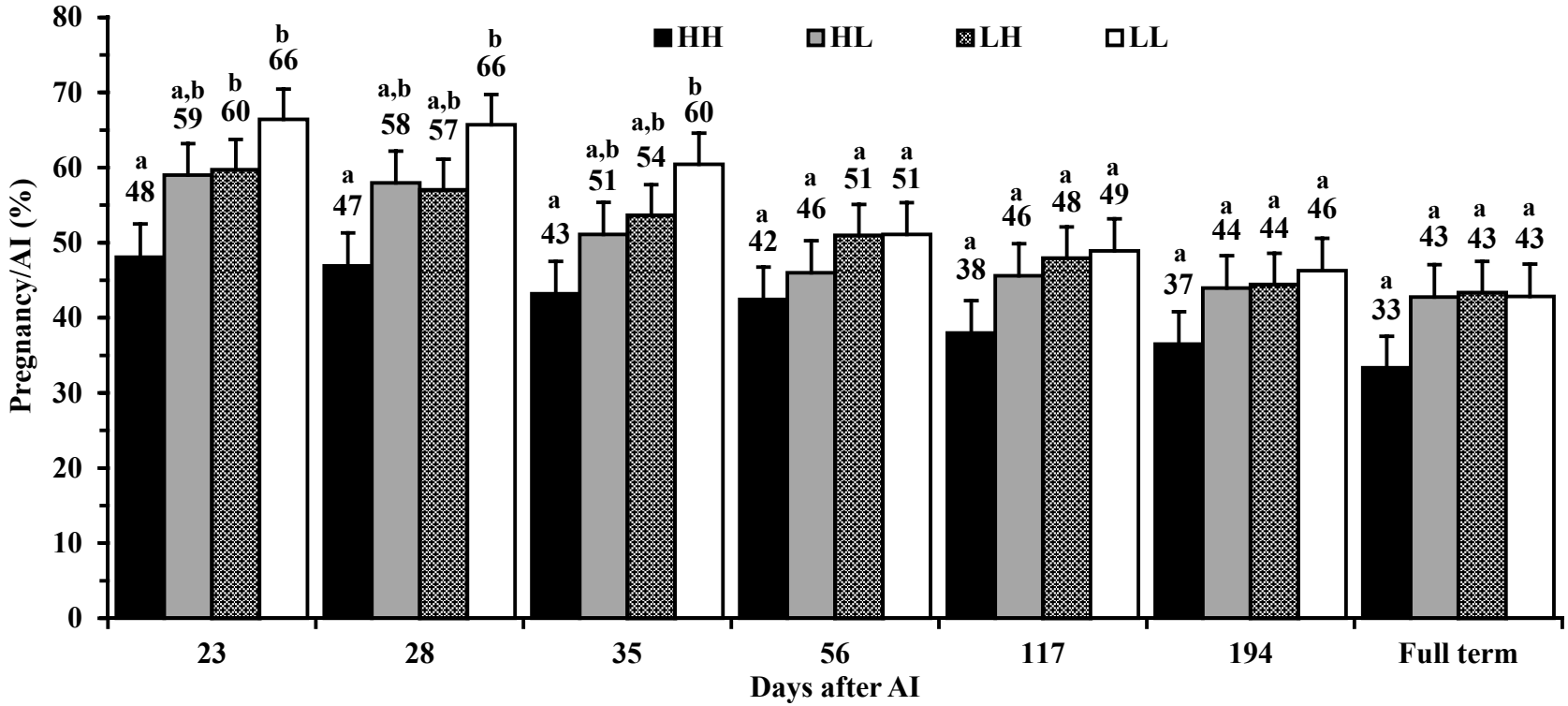

Figure 8.11. Effect of treatment on pregnancy per AI in different days after AI. Data are shown as mean ± SEM. [a,b,c] Different letters within a day after AI represent differences of least square means ($P < 0.05$).

Effect of treatment and double ovulation on fertility parameters

Treatment had an effect ($P < 0.05$) on P/AI on day 23 post-AI (Table 3.2/ Figure 3.11). Cows treated with H/H had lower P/AI compared to cows treated with L/H and L/L and tended ($P < 0.10$) to be lower compared to cows treated with H/L (Table 3.2). Double ovulation also had an effect on P/AI on day 23 post-AI (Table 3.3). Cows with double ovulation had greater P/AI on day 23 post-AI

compared to cows with single ovulation (Table 3.3). Thus, treatment differences in P/AI day 23 appears to be due the differences in double ovulation rates. Furthermore, cows treated with L/L had greater percentage of pregnancy losses between day 35 and 56 post-AI compared to H/H and a trend to greater losses compared to L/H (Table 3.2). It appears that the increased percentage of double ovulation for cows treated with L/L had an impact on pregnancy losses during day 35 and 56 of gestation. However, cows with single ovulation in the same treatment still had a high number of cows with pregnancy loss (Table 3.4).

Table 8.2. Effect of treatments on pregnancy per AI (P/AI) at day 23 post-AI, pregnancy losses during gestation, gestation length, calving, twining, stillborn and gender rates.

	Treatments			
	HH	**HL**	**LH**	**LL**
P/AI 23 days post-AI, % (n/n)	48.1^{a}*(62/129)	59.0^{ab}* (82/139)	59.7^{b} (89/149)	66.4^{b} (93/140)
Pregnancy losses				
23 to 28 days post-AI[1], % (n/n)	1.6 (1/62)	1.2 (1/81)	4.5 (4/89)	1.1 (1/93)
28 to 35 days post-AI[1], % (n/n)	6.6 (4/61)	11.4 (9/79)	3.6 (3/84)	7.7 (7/91)
35 to 56 days post-AI[1], % (n/n)	1.8^{a} (1/57)	10.0^{ab} (7/70)	4.9^{ab}*(4/81)	14.5^{b}* (12/83)
56 to 117 days post-AI[1], % (n/n)	7.5 (4/53)	0 (0/62)	2.8 (2/72)	2.9 (2/69)
117 to 194 days post-AI[1], % (n/n)	0 (0/46)	0 (0/58)	5.9 (4/68)	3.1 (2/64)
194 to 265 days post-AI[1], % (n/n)	8.7 (4/46)	1.8 (1/57)	1.6 (1/63)	6.6 (4/61)
Total[2], % (n/n)	25.0 (14/56)	24.3 (18/74)	22.5 (18/80)	32.9 (28/85)
Cows culled or died pregnant during experiment due to non-reproductive reasons, n	6	8	9	8
Calving[3], % (n/n)	34.1*(42/123)	42.7 (56/131)	44.3*(62/140)	43.2 (57/132)
Gestation length, days ± SEM	280.8 ± 0.7	280.2 ± 0.7	279.8 ± 0.6	279.3 ± 0.6
Twins, % (n/n)	4.8^{a}*(2/42)	14.3^{ab}*(8/56)	11.3^{ab} (7/62)	19.3^{b} (11/57)
Female calf, % (n/n)	47.7 (21/44)	42.2 (27/64)	37.7 (26/69)	41.2 (28/68)
Stillborn, % (n/n)	0 (0/44)	1.6 (1/64)	0 (0/69)	5.9 (4/68)
Total live calves / total of cows inseminated, % (n/n)	35.8^{d} (44/123)	48.1^{e} (63/131)	49.3^{e} (69/140)	48.5^{e} (64/132)

[1]Cows that were culled or died pregnant due to non-reproductive reasons during this stage of gestation were removed from this calculation.
[2]Cows that were culled or died pregnant during the experiment period were removed from this calculation.
[3]Calving rate = cows that calved divided by the cows inseminated, removing the cows that were culled or died pregnant during the experiment period.
[a-c] Means and percentages within a row with different letters differ ($P \leq 0.05$).
[d-e] Percentages within a row with different had a tendency to differ ($P < 0.09$).
*Percentages within a row with asterisk had a tendency to differ ($P \leq 0.10$).

Table 8.3. Effect of number of ovulation and side of double ovulation on pregnancy per AI (P/AI) at day 23 post-AI, pregnancy losses during gestation, gestation length and calving, twining, stillborn and gender rates.

Parameter	Single ovulation	Double Ovulation			*P* for single vs. double ovulation	Overall
		Unilateral	Bilateral[1]	Total		
P/AI 23 days post-AI, % (n/n)	50.9[a] (189/371)	72.2[b] (65/90)	73.0[b] (65/89)	72.6 (130/179)	<0.001	58.0 (319/550)
Pregnancy losses						
23 to 28 days post-AI[1], % (n/n)	2.1 (4/188)	3.1 (2/65)	1.5 (1/65)	2.3 (3/130)	0.88	2.5 (8/318)
28 to 35 days post-AI[1], % (n/n)	8.2 (15/183)	4.8 (3/62)	7.9 (5/63)	6.4 (8/125)	0.31	7.5 (23/308)
35 to 56 days post-AI[1], % (n/n)	4.2[a] (7/168)	16.9[b] (10/59)	8.8[ab] (5/57)	12.9 (15/116)	0.02	7.7 (22/284)
56 to 117 days post-AI[1], % (n/n)	2.6 (4/156)	4.5 (2/44)	3.9 (2/51)	4.2 (4/95)	0.26	3.2 (8/251)
117 to 194 days post-AI[1], % (n/n)	1.4 (2/146)	7.7 (3/39)	2.2 (1/46)	4.8 (4/85)	0.21	2.6 (6/231)
194 to 265 days post-AI[1], % (n/n)`	4.2 (6/142)	5.6 (2/36)	2.3 (1/44)	3.8 (3/80)	0.88	4.1 (9/222)
Total[2], % (n/n)	21.8[a] (38/174)	39.3[b*] (22/56)	25.9[ab*] (14/58)	31.6 (36/114)	0.05	25.7 (74/288)
Cows culled or died pregnant during experiment due to non-reproductive reasons, n	15	9	7	16	-	31
Calving[3], % (n/n)	38.2[a] (136/356)	42.0[ab] (34/81)	52.4[b] (43/82)	47.2 (77/163)	0.055	41.0 (213/519)
Gestation length, days ± SEM	280.6[a] ± 0.4	279.3[ab] ± 0.9	278.7[b] ± 0.7	278.9 ± 0.6	0.04	280.0 ± 0.3
Twins, % (n/n)	0[a] (0/136)	23.5[b] (8/34)	46.5[c] (20/43)	36.4 (28/77)	<0.001	13.1 (28/213)
Female calf, % (n/n)	40.4 (55/136)	47.6 (20/42)	42.8 (27/63)	44.8 (47/105)		42.3 (102/241)
Stillborn, % (n/n)	0 (0/136)	4.8 (2/42)	4.8 (3/63)	4.8 (5/105)		2.1 (5/241)
Total live calves / total of cows inseminated, % (n/n)	38.2[a] (136/356)	49.4[a] (40/81)	73.2[b] (60/82)	61.3 (100/163)	<0.001	45.5 (236/519)

[1]Three cows had triple ovulation with double ovulation in one ovary and single ovulation in the other ovary. These cows were considered to have bilateral ovulation.

[2]Cows that were culled or died pregnant due to non-reproductive reasons during this stage of gestation were removed from this calculation.

[3]Cows that were culled or died pregnant during the experiment period were removed from this calculation.

[4]Calving rate = cows that calved divided by the cows inseminated, removing the cows that were culled or died pregnant during the experiment period.

[a-c] Means and percentages within a row with different letters differ ($P \leq 0.05$).

*Percentages within a row with asterisk had a tendency to differ ($P = 0.08$).

Table 8.4. Effect of treatments on pregnancy per AI (P/AI) at day 23 post-AI, pregnancy losses during gestation, gestation length, calving, twining, stillborn and gender rates.

	Treatments							
	HH		HL		LH		LL	
	Single ovulation	Double ovulation	Single ovulation	Double ovulation	Single ovulation	Double ovulation	Single ovulation	Double ovulation
P/AI 23 days post-AI, % (n/n)	44.6^{aA} (50/112)	68.8^{B} (11/16)	49.5^{a} (45/91)	76.1^{b} (35/46)	56.1^{B} (55/98)	66.0^{B} (33/50)	55.7^{a} (39/70)	76.5^{b} (52/68)
Pregnancy losses								
23 to 28 days post-AI[1], % (n/n)	0 (0/50)	9 (1/11)	0 (0/44)	2.9 (1/35)	7.2 (4/55)	0 (0/33)	0 (0/39)	1.9 (1/52)
28 to 35 days post-AI[1], % (n/n)	8 (4/50)	0 (0/10)	18.6 (8/43)	2.9 (1/34)	3.9 (2/51)	3.1 (1/32)	2.6 (1/39)	12 (6/50)
35 to 56 days post-AI[1], % (n/n)	0 (0/46)	10 (1/10)	2.9 (1/35)	15.2 (5/35)	2.0 (1/49)	9.7 (3/31)	13.2 (5/38)	14.0 (6/43)
56 to 117 days post-AI[1], % (n/n)	6.8 (3/44)	12.5 (1/8)	0 (0/34)	0 (0/27)	0 (0/46)	8 (2/25)	3.1 (1/32)	2.8 (1/36)
117 to 194 days post-AI[1], % (n/n)	0 (0/39)	0 (0/6)	0 (0/33)	0 (0/24)	4.5 (2/44)	8.7 (2/23)	0 (0/30)	6 (2/33)
194 to 265 days post-AI, % (n/n)	7.7 (3/39)	16.7 (1/6)	3 (1/33)	0 (0/23)	2.4 (1/41)	0 (0/21)	3.4 (1/29)	6.5 (2/31)
Total[2], % (n/n)	21.7 (10/46)	44.4 (4/9)	23.8 (10/42)	23.3 (7/30)	20 (10/50)	27.6 (8/29)	22.2 (8/36)	38.3 (18/47)

[1]Cows that were culled or died pregnant due to non-reproductive reasons during this stage of gestation were removed from this calculation.

[2]Cows that were culled or died pregnant during the experiment period were removed from this calculation.

[3]Calving rate = cows that calved divided by the cows inseminated, removing the cows that were culled or died pregnant during the experiment period.

[a-c] Means and percentages within a row with different letters differ ($P \leq 0.05$).

[d-e] Percentages within a row with different had a tendency to differ ($P < 0.09$).

*Percentages within a row with asterisk had a tendency to differ ($P \leq 0.10$).

Cows with unilateral double ovulations also had greater percentage of pregnancy losses between days 35 and 56 post-AI compared to cows with single ovulation or cows with bilateral double ovulation (Table 3.3). Overall percentage of pregnancy losses between day 23 post-AI to calving did not differ between treatments (Table 3.2). However, cows with unilateral double ovulation had greater percentage of pregnancy losses from day 23 post-AI to calving compared to cows with single ovulation or cows with bilateral double ovulation (Table 3.3). Percentage of cows calving after treatment tended ($P < 0.10$) to be greater for cows treated with L/H compared to cows treated to H/H (Table 3.2). Cows treated with H/H had a lower proportion of twin births compared to cows treated with L/L and tended to be lower compared to cows treated with H/L (Table 3.2). Cows treated with H/H had fewer live calves born per cows inseminated compared to the other treatments (Table 3.2). Cows treated with L/L had greater P/AI on days 23, 28 and 35 post-AI; however, on day 56 and beyond P/AI was similar between L/L and H/H due to the greater pregnancy losses in L/L between days 35 and 56 of gestation (Figure 3.10). In addition, percentage of cows with full term births were similar between L/L and H/H (Figure 3.10 and Table 3.2). These results are in accordance with our double ovulations response for treatments.

Cows with bilateral double ovulation had greater percentage of full term births compared to cows with single ovulation (Table 3.3). Proportion of twin births was greater for cows with bilateral double ovulation compared to cows with unilateral double ovulation (Table 3.3). Gestation length was smaller for cows with bilateral double ovulation compared to cows with single ovulation (Table 3.3). Cows with bilateral double ovulation had a greater proportion of live calves born per total cows inseminated compared to cows with unilateral double ovulation or cows with single ovulation (Table 3.3).

Table 8.5. Effect of low vs. high serum concentrations of progesterone (P4) during different phases of the ovulatory follicle development on pregnancy per AI (P/AI) at day 23 post-AI, pregnancy losses during gestation, gestation length, calving, twining, stillborn and gender rates.

	Treatments combined based on levels of P4 during different phases of the ovulatory follicle development					
	Pre-dominance Phase			Dominance Phase		
Parameter	High P4 (HL + HH)	Low P4 (LH + LL)	*P*	High P4 (LH + HH)	Low P4 (HL + LL)	*P*
P/AI 23 days post-AI, % (n/n)	53.7 (144/268)	63.0 (182/289)	0.09*	54.3 (151/278)	62.7 (175/279)	0.05
Pregnancy losses						
23 to 28 days post-AI[1], % (n/n)	1.4 (2/143)	2.7 (5/182)	0.51	3.3 (5/151)	1.1 (2/174)	0.18
28 to 35 days post-AI[1], % (n/n)	9.3 (13/140)	5.7 (10/175)	0.21	4.8 (7/145)	9.4 (16/170)	0.12
35 to 56 days post-AI[1], % (n/n)	6.3 (8/127)	9.8 (16/164)	0.24	3.6 (5/138)	12.4 (19/153)	0.02
56 to 117 days post-AI[1], % (n/n)	3.5 (4/115)	2.8 (4/141)	0.80	4.8 (6/125)	1.5 (2/131)	0.18
117 to 194 days post-AI[1], % (n/n)	0 (0/104)	4.5 (6/132)	0.97	3.5 (4/114)	1.6 (2/122)	0.39
194 to 265 days post-AI[1], % (n/n)	4.9 (5/103)	4.0 (5/124)	0.61	4.6 (5/109)	4.2 (5/118)	0.70
Total[2], % (n/n)	24.6 (32/130)	27.9 (46/165)	0.54	23.5 (32/136)	28.9 (46/159)	0.39
Cows culled or died pregnant during experiment due to non-reproductive reasons, n	14	17	-	15	16	-
Calving[3], % (n/n)	38.6 (98/254)	43.8 (119/272)	0.30†	39.5 (104/263)	42.9(113/263)	0.38
Gestation length, days ± SEM	280.5 ± 0.5	279.6 ± 0.4	0.13	280.2 ± 0.5	279.8 ± 0.5	0.53
Twins, % (n/n)	10.2 (10/98)	15.1 (18/119)	0.24	8.7 (9/104)	16.8 (19/113)	0.06
Female calf, % (n/n)	44.4 (48/108)	39.4 (54/137)	-	41.6 (47/113)	41.7. (55/132)	-
Stillborn, % (n/n)	0.9 (1/108)	2.9 (4/137)	-	0 (0/113)	3.8 (5/132)	-
Total live calves / total of cows inseminated, % (n/n)	42.1 (107/254)	48.9 (133/272)	0.18	42.9 (113/263)	48.3 (127/263)	0.36

[1]Cows that were culled or died pregnant due to non-reproductive reasons during this stage of gestation were removed from this calculation.

[2]Cows that were culled or died pregnant during the experiment period were removed from this calculation.

[3]Calving rate = cows that calved divided by the cows inseminated, removing the cows that were culled or died pregnant during the experiment period.

*Interaction between parity category and P4 during the pre-dominance was observed for P/AI 23 days post-AI (P = 0.15). Multiparous cows with low P4 during the pre-dominance phase of follicle development had greater (P <0.01) P/AI 23 days post-AI compared to multiparous cows with high P4 during pre-dominance (51.7 % vs. 65.4 %; respectively).

†Interaction between service category and P4 during the pre-dominance was observed for P/AI 23 days post-AI (*P* = 0.09). First AI cows with low P4 during pre-dominance period had greater (*P* = 0.04) calving rate compared to first service cows with high P4 during pre-dominance phase.

Effect of high vs. low serum P4 during pre-dominance or dominance phase of the ovulatory follicle development with treatments combined

In Table 3.5, treatments were combined to determine the effects of low or high serum P4 on the pre-dominance phase and dominance phase. Cows with low serum P4 during pre-dominance phase (L/H and L/L) had a trend for greater P/AI compared to cows with high serum P4 in the same period (H/H and L/H). However, there were no other differences in pregnancy losses, calving rate, gestation length, twin births, and total live calves born per cow inseminated between low and high serum P4 during pre-dominance phase.

Cows with low serum P4 during the dominance phase (H/L and L/L) had also greater P/AI on day 23 post-AI compared to cows with high serum P4 (H/H and L/H) (Table 5). However, cows with low P4 during the dominance phase had greater ($P < 0.05$) percentage of pregnancy loss between days 28 and 56 after AI compared to cows with high P4 during the dominance phase (20.7 vs. 8.3 %; respectively). Following this period of gestation, there were no differences in percentage of pregnancy loss, calving rate, gestation length and total live calves born per total cows inseminated for cows with low vs. high serum P4 during the dominance period. There was also a trend for greater percentage of twin births for cows with low P4 during the dominance phase compared to cows with high P4.

Cows with single ovulation and low P4 during the dominance phase (H/L and L/L combined) had similar (P = 0.68) P/AI at day 23 compared to cows with single ovulation and high P4 (H/H and L/H combined; 52.2 % vs. 50.0 %; respectively). However, single ovulating cows with low P4 during the dominance phase had greater (P = 0.02) pregnancy loss between days 28 and 56 post-AI compared to cows with high P4 and single ovulation (18.3 % vs. 6.9 %; respectively). Yet, total pregnancy loss (P = 0.75; 23.1 vs. 20.8. %, respectively) and calving rate

(P = 0.80; 38.5 % vs. 36.9 %; respectively) was not different in cows with single ovulation with low versus high P4 during the dominance phase.

DISCUSSION

The main objective of the present study was to determine the effects of inducing high or low serum levels of P4 during different stages of the ovulatory follicle growth on fertility of lactating dairy cows. Our hypothesis was that reduced serum concentrations of P4 during the dominance period of the ovulatory follicle have the potential to decrease P/AI and increase pregnancy loss during gestation. Results indicated that cows with low circulating P4 during dominance had greater P/AI on day 23 post-AI. This result was related to the increased double ovulation rate that resulted in greater percentage of cows pregnant at day 23 post-AI. However, a greater percentage of cows with low P4 during dominance period of ovulatory follicle development had pregnancy loss between days 28 and 56 of gestation. Greater pregnancy losses during this period was observed not only in double ovulation cows but also in single ovulation cows with low P4 during dominance. These results partially support our hypothesis. Cows with unilateral double ovulation had greater percentage of pregnancy loss during this stage of gestation. Although cows with low P4 during the dominance period had greater P/AI 23 day post-AI, percentage of cows calving were similar for cows compared to cows with high P4 during the dominance phase because of the greater percentage of pregnancy losses between days 28 and 56 of gestation.

Several studies during the last decade investigated the effects of serum levels of P4 during the growth of the ovulatory follicle on fertility of cattle [21-25]. Although there is strong evidence that low levels of P4 during ovulatory follicle development may play a role on

infertility of lactating dairy cows, the mechanisms involved are not stablished. The majority of past studies showed a positive relationship between circulating concentrations of P4 during ovulatory follicle development and fertility of lactating dairy cows [21-25]. A recent study compared P/AI of cows inseminated after ovulation of ovulatory follicles of first wave, that developed under low P4, compared to ovulatory follicles of second wave, which developed under greater P4 [21]. Cows with ovulatory follicles from second wave had greater P/AI compared to cows with ovulatory follicles from first wave [21]. Previous work that used a synchronization of ovulation program have determined that cows without a CL at beginning of the program have lower P/AI compared to cows with a CL, in diestrus [23,38]. Furthermore, cows without a CL at the initiation of a synchronization of ovulation program that were supplemented with P4 (2 CIDRs) had greater P/AI compared to cows without CL and similar P/AI compared to cows with CL (in diestrus) [23,38]. A recent meta-analysis of 25 controlled studies that used synchronization of ovulation protocols also identified a benefit of supplement P4 during the growth of the ovulatory follicle on P/AI [22]. Yet, the improvements of fertility with supplementation of P4 could be not only due to the enhancement of P4 but also due a better synchronization of cows to the protocol.

Therefore, in order to test our hypothesis, the present study reduced factors that could have influenced our outcomes. The design of the present study controlled the initiation of the ovulatory follicular wave and manipulated serum concentrations of P4 during two different phases of follicle development. All cows used were in a similar period of the cycle and had similar follicular wave duration, initiating on day 1 of treatment and completing on treatment day 11 with ovulation. Controlling the wave length was an important factor since follicles that develop under low circulating P4 with long duration of dominance (persistent follicle) have

lower fertility compared to cows with greater P4 and short duration of dominance [31,39]. The reduction of fertility in oocytes from persistent follicles have been attribute to LH overstimulation causing germinal vesicle breakdown [29]. It also has been demonstrated that cows with ovulatory follicle from the first follicular wave of the cycle have lower P/AI compared to cows with ovulatory follicles from second follicular wave [21]. Thus, the design of the present experiment only used cows with ovulatory follicles from second follicular wave. In addition, all cows at beginning of treatment were cycling (with a CL) since cows without a CL at initiation of the follicular wave have lower P/AI compared to cows with a CL [23,38]. Finally, all cows used were in a good health condition Therefore, the present controlled experiment reduced the probability of having other confounding factors that could have influenced our results.

Results showed that double ovulation rate was extremely increased after treatments with low P4 during ovulatory follicle development. In addition, double ovulation had a major influence on fertility outcomes. Previous research reported strong evidence of the effect of low P4 on increased incidence of double ovulation in cattle. Recent studies that manipulated low vs. high circulating P4 with a controlled follicular wave similar to the present experiment showed that cows with low P4 during entire follicle development had a greater percentage of double ovulations compared to cows with high P4 [26,40]. Heifers and cows with a follicular wave in the absence of a CL, thus, very low circulating P4, had increased co-dominant follicles and double ovulation [41]. This study suggested a role for the enhancement of LH pulses due to low circulating P4 on increased incidence of co-dominant follicles [41]. In agreement, Lopez et al. (2004)[42] showed that cows with double ovulation had low circulating P4 and greater circulating LH and FSH prior to deviation compared to cows with single ovulations. In the current study, low circulating P4 also increased incidence of multiple ovulations in lactating

dairy cows. High circulating P4 had also an opposite effect on cows with history of double ovulations and may be an efficient way to reduce double ovulation incidence in cows with history of double ovulation. Although our results are in agreement with earlier studies regarding the effect of low circulating P4 on double ovulation, the percentage of cows with double ovulation was greater than previously reported (18.6 % [26], 20.6 % [24], 25.0 % [40], 26.8 % [43]). The high average milk production for cows used in the experiment (Farm average ~ 42 Kg/day) might be related to the greater incidence of double ovulation previously reported. Lopez et al. [8] reported 45.3 % of double ovulation for cows with milk production between 45 and < 50 Kg/day and 51.6 % for cows with milk production ≥ 50 Kg/day. High milk production was associated with the increased metabolic clearance rate of steroid hormones due to greater feed intake and greater blood flow through the liver [2-6].

The high incidence of double ovulations in cows induced to have high P4 only during the first 4 days of the follicular wave (H/L treatment) was not anticipated. It was expected that cows treated with L/H had greater double ovulations compared to cows treated with H/L but double ovulation rate was similar between these two treatments. It was previously suggested that low circulating P4 would increase LH pulses and delay the decrease of circulating FSH to basal prior to deviation causing an increase on frequency of double ovulation in cattle [44]. A key event for follicle deviation is the acquisition of LH receptors in the granulosa cells [37,45-47] . Although 4 days after GnRH treatment was considered day of deviation in the currently experiment based on previous data [37], there are some variation on day of acquisition of LH receptors by granulosa cells [46-49]. This time difference of deviation between cows might be the reason for similar percentage of cows with double ovulation in treatment L/H and H/L. Previous studies determined that deviation happens in less than 8 h [50,51], which is a very narrow period. Cows treated with

H/L with double ovulation might had ovulatory follicle deviation after day 4 of treatment. It is also unknown if high circulating P4 during the pre-dominance phase delays the selection process when compared to low P4. Cows treated with low P4 during the entire follicular wave might had more chances to have low circulating P4 close to selection.

The high percentage of double ovulation positively influence P/AI on day 23 post-AI. It is obvious to think that the presence of two oocytes at time of fertilization would increase probability of pregnancy. However, it is not known if the increase in P/AI 23 day post-AI was caused due to a greater fertilization rate or due to a greater embryonic survival between fertilization and day 23 post-AI or both. A previous report did not find differences in fertilization rate between cows with single or multiple ovulations at day 6 to 8 post-mating (~ 80 %; [52]). In addition, there was no differences in percentage of embryos classified as normal or abnormal and oocytes classified as unfertilized days 6 to 8 post-mating [52]. Past report also found greater P/AI in cows with double ovulation compared to cows with singe ovulation [53]. In addition to high P/AI at day 23 post-AI, cows with double ovulation had greater pregnancy losses between days 35 and 56 post-AI compared to single ovulation cows, primarily due to losses in cows with unilateral double ovulations. Echternkamp [52] found a greater incidence of dead or degenerated fetus on day 52 of gestation for cows with triple ovulations or more and suggested that embryo death occurred approximately day 35 of gestation based on fetal development. Lopez-Gatius [43] also identified cows with twins with a greater risk of suffering pregnancy loss (3.1 times greater) compared to singletons between days 38 and 90 of gestation. In a later study, Lopez-Gatius [54] found 28.4 % of pregnancy losses in twin pregnancies between approximately days 30 and 60 post-AI. In a study with large number of heifers and cows, twins had greater percentage of pregnancy losses than singletons in cows [53]. In heifers, unilateral twin pregnancies had 2- to 3-

fold greater pregnancy losses between days 75 to 135 of gestation compared to bilateral twin pregnancies.

One of the causes of pregnancy loss for unilateral twining suggested was crowding of 2 embryos or fetuses within one horn [52,53]. Although single dead fetus expulsion or resorption have been reported in cattle [55,56], the gravid uterus of bovine with twins does not appear to have the capacity to selectively expel only one dead fetus as reported in mares [57] or selectively resorb as reported in swine [58]. From 28 to 30 days post-insemination, placentation process starts. Incidence of placental anastomosis in twin pregnancies in cattle is > 90 % based on the frequency of freemartin in twin births. The vascular anastomosis appears to preclude the expulsion or absorption of the dead fetus, which may lead to the death of the other viable fetus. Unfortunately, in the current study, twin data was not collected during pregnancy to determine if there was a reduction of embryo number during this period. Although circulating concentrations of PSPB is greater in twin pregnancies compared to singleton [56], it did not provide a high specificity and sensitivity to predict twin birth. If both oocytes of cows with unilateral double ovulation were at the same location at time of fertilization and one was fertilized there was a high probability that the other would also be fertilized. In this case, cows in the present experiment with unilateral double ovulation may had a greater percentage of one embryo loss compared to cows with bilateral double ovulations.

Lopes-Gatius found that unilateral twin pregnancies had a greater risk (3.45 times greater) to suffer losses of one embryo compared to bilateral twin pregnancies [54]. However, it was not determined if losses occurred prior to placental anastomosis. It has been reported that cows with losses between days 31 and 60 of gestation had lower circulating PSPB already on day 24 of gestation (add Heathers poster)[59]. Interestingly, our results indicated that pregnant cows

with unilateral double ovulations had lower circulating concentration of PSPS on days 23 and 28 of gestation compared to cows pregnant with bilateral double ovulation and similar compared to pregnant cows with single ovulation. This result could be due to the presence of just one viable embryo or due to two embryos in the same horn with impaired placentation. PSPB and other pregnancy associated glicoproteins (PAGs) or pregnancy specific proteins (PSP) are produced by binucleate cells (BNC). During placentation, the BNC are critical for two main functions: formation of the fetomaternal syncytium critical for implantation and subsequent placentomal formation; and production and secretion of other proteins and steroid hormones such as somatomammotropin hormone 1 (CSH1 or placental lactogen) and progesterone. Taken together, this results suggests that the placentation process in unilateral twins may be compromised.

Lower percentage of twin births in unilateral double ovulation cows compared to cows with bilateral double ovulations also suggests a high percentage of one embryo losses in unilateral double ovulation cows. The great majority of twin births has been determined to be due double ovulation (approximately 95%). In agreement, our data did not have any twin birth from cows with single ovulation. Low P4 treatments increased percentage of calves per cow inseminated due to greater percentage of double ovulations and the increased percentage of twin births. However, twin births in dairy cows is undesirable due to the greater incidence of complications peripartum (), that can bring more costs to the producers.

Besides the greater percentage of losses most likely due to unilateral twins, cows with low P4 during the dominance period of the ovulatory follicle development also had a greater percentage of pregnancy losses in single ovulation cows. Low circulating P4 during the growth of the ovulatory follicle has been related to an increase in pregnancy losses between days 29 and 60 post-AI in lactating dairy cow [21,24]. This result partially supports our hypothesis. The

mechanisms involved in losses during this period are not understood. As previously discussed, placentation takes place during this period of gestation, and it might also be linked to pregnancy loss of cows with low P4 during dominance of the ovulatory follicle and single ovulation.

We initially hypothesized that cows with low P4 during the dominance period and single ovulation would have low P/AI at day 23 post-AI compared to cows with high P4. However, cows with low P4 had similar P/AI at day 23 post AI compared to cows with high P4 during dominance and single ovulation. The perfect synchronization of all cows used in the present study with similar ovulatory follicular wave duration may have influenced positively in our results of P/AI at day 23 post-AI for cows with single ovulation. An earlier study that also used only synchronized animals did not find differences on embryo quality 6 days after AI between cows with low versus high progesterone during ovulatory follicular development [26]. In addition, it was identified that cows with low P4 during a synchronized ovulatory follicular wave had greater incidence of short luteal phase (before day 10 of the subsequent cycle) compared to cows with high P4 (25 % vs. 0 %; respectively [40]). In disagreement with this study, our results did not have occurrence of short luteal phase. Only 3 % of all cows treated was detected with complete luteolysis 14 and 16 days after AI, and the majority of cows were detected 20 or 23 days after AI. This result suggests that CL lifespan does not play a role on pregnancy losses of cows with low P4 during dominance of ovulatory follicle development.

The increase in serum concentrations of PSPB from basal (average serum concentration of PSPB on days 16 and 20) to day 23 post-AI was indicated as a good marker for pregnancy in the present study. Circulating PSPB has been used as a marker for pregnancy on and after 28 days post-AI with high specificity and sensibility. To our knowledge, this is the first time that circulating levels of PSPB have been used as a maker of pregnancy on day 23 post-AI. Data from

our laboratory (J. R. Pursley, unplub. data) and others [59] have shown that PSPB levels increase in pregnant cows around day 22 after AI. An early and accurate diagnostic method for pregnancy would be of extremely important for dairy producers to rebreed non-pregnant cows in an earlier manner.

In conclusion, circulating concentrations of P4 during ovulatory follicle wave development affected diameter of the ovulatory follicle and rate of double ovulations. Cows with a history of double ovulation during pre-treatment had a greater probability of double ovulation regardless of treatment; however, induction of high P4 during ovulatory follicular development appears to reduce the risks of double ovulation in these cows. The hormonal environment of the growing pre-ovulatory follicle is a regulator of subsequent pregnancy loss. Low P4 during growth of the pre-ovulatory follicle created greater losses during post-attachment period to 56 days post-AI. Most losses post-attachment were likely due to unilateral twins.

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CHAPTER 9

EFFECT OF PROSTAGLANDIN $F_{2\alpha}$ DURING EARLY CORPUS LUTEUM DEVELOPMENT ON CIRCULATING CONCENTRATIONS OF PROGESTERONE IN BREEDING AGE DAIRY HEIFERS

Manuscript will be submitted to Theriogenology

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ABSTRACT

As part of an overarching objective to create a low progesterone (P4) environment during growth of the pre-ovulatory follicle in heifers to determine the effect of low P4 on fertility parameters, we tested the effect of $PGF_{2\alpha}$ on early corpus luteum (CL) function. Dairy heifers between 12 and 13 mo of age were pre-synchronized to ensure all heifers were on d 6 of the estrous cycle at the start of the Ovsynch program. Only heifers that responded to the following strategy with CL regression and ovulation determined using ultrasound were utilized: 0.5 mg cloprostenol ($PGF_{2\alpha}$) –2 d– 0.1 mg GnRH –6 d– GnRH (G1; 1st GnRH of Ovsynch). Heifers (n=159) that responded to the pre-treatment were randomly assigned to 4 groups: high P4 control (HPC), low P4 control (LPC), and treatments PG2 and PG3. Heifers in LPC, PG2 and PG3 received $PGF_{2\alpha}$ 1 d after G1 to regress all d-7 CL. Heifers from these groups had only 1 new CL growing during the treatment period. Groups PG2 and PG3 received treatments of $PGF_{2\alpha}$ 2 and 3 d after G1, respectively. HPC did not receive $PGF_{2\alpha}$ on d 1 following G1, thus had 2 CL (a mature and a new CL) growing during the treatment period. Starting on d 7 after G1, heifers received $PGF_{2\alpha}$ –1 d– $PGF_{2\alpha}$ –1 d– GnRH (G2). Blood samples were collected in all heifers on d 7 (n=157) and in a subset of heifers on d 1, 2, 3, 4 (n=82) after G1 to measure serum P4. Pre-ovulatory follicle size at G1 (13.0 ± 0.1 mm; P=0.53) and mean serum P4 on d 1 (3.62 ± 0.11 ng/mL; P = 0.46) did not differ between treatments. HPC treated heifers had greater (P<0.001) mean serum P4 compared to LPC, PG2 and PG3 on d 2, 3, 4, and 7. On d 2, 3 and 4, mean serum P4 of LPC, PG2 and PG3 treated heifers did not differ (P>0.10). On d 7, LPC heifers had greater (P<0.001) serum P4 compared to PG2 and PG3 heifers. Mean ± SEM serum P4 on d 7 after G1 was 8.43 ± 0.39, 2.55 ± 0.36, 1.58 ± 0.20, and 1.21 ± 0.15 ng/mL for HPC, LPC, PG2, and PG3, respectively. Percentage of heifers with P4 < 0.50 ng/mL on d 7 was greater (P<0.05) for LPC, PG2 and PG3 (27, 32 and 26 %,

respectively) compared to HPC (0 %). In summary, $PGF_{2\alpha}$ treatments during early CL development reduced circulating concentrations of P4 7 d after G1 compared with both HPC and LPC.

INTRODUCTION

Prostaglandin $F_{2\alpha}$ is a luteolytic agent in cattle that is largely used to induce luteolysis and estrus in different strategies of cattle reproductive management [1-4]. These strategies use exogenous im administration of $PGF_{2\alpha}$ or its analogues to induces complete regression of bovine corpus luteum (CL) after d 5 of the estrous cycle [5-9]. High-affinity $PGF_{2\alpha}$ receptors (FP receptors) are present in the bovine corpus luteum (CL) by 2 d after ovulation (approximately d 3 of the estrous cycle), and with similar concentrations of FP receptors as a mature CL (d 11) [10,11]. However, prior to d 5 of the estrous cycle, the bovine CL appears to be refractory to $PGF_{2\alpha}$ or its analogues [1,2,4,12-15]. Different routes of administration and doses of $PGF_{2\alpha}$ failed to induce complete luteolysis of a newly formed CL prior to d 5 of the estrous cycle [1,2,4,12-15].

Yet, during this period, $PGF_{2\alpha}$ appeared to have a negative effect on CL function. Repeated treatments of $PGF_{2\alpha}$ [4,16-20], a double dose of $PGF_{2\alpha}$ within the first 5 d of the estrous cycle [15,17,19], or a single dose on d 4 or 5 [13,18] impacted the early CL through a reduction of circulating concentrations of progesterone (P4) in both cows and heifers. It appears that CL responsiveness to $PGF_{2\alpha}$ is dependent on frequency and amplitude (dose) of the luteolytic stimulus and age of the CL [14]. Different experiment models used $PGF_{2\alpha}$ during the 4 d of the estrous cycle to test the effects of low serum concentrations of P4 on follicle development [18,20] and embryo development [16] . Nevertheless, to our knowledge, all studies investigated

the effects of $PGF_{2\alpha}$ or its analogues administered during the first 5 d of the estrous cycle on a newly formed CL. There is a lack of studies that investigated the effect of $PGF_{2\alpha}$ or its analogues on the early function of a CL formed during the diestrus period of the estrous cycle.

Therefore, the present study tested the effect of a single label dose of a $PGF_{2\alpha}$ analogue, cloprostenol sodium, administered im during the first 2 d of CL life span on the function of a newly formed CL during diestrus (~ d 7 of the estrus cycle). The main objective was to use $PGF_{2\alpha}$ during the early period of CL development to reduce serum P4 to sub-normal luteal levels during growth of the pre-ovulatory follicle yet maintaining CL function until controlled induced luteolysis at time of $PGF_{2\alpha}$ of Ovsynch. The overarching objective was to create a model of low P4 environment during growth of the pre-ovulatory follicle in heifers to determine the effect of low serum P4 on fertility. We hypothesized that a single label dose of $PGF_{2\alpha}$ administered im in dairy heifers on d 1 or 2 after ovulation would reduce serum P4 6 d after ovulation.

MATERIALS AND METHODS

Heifers, housing, feeding, products and detection of estrus

This study was conducted from May and August of 2012 on a commercial dairy farm in Michigan (Green Meadow Farms, Elsie, MI). Holstein heifers used in this study were between 12 and 13 months of age during treatment and were housed in free stall barns with free access to water and fed a TMR 1x/d. Farm trained personnel monitored and recorded signs of estrus three times daily (morning, afternoon and evening). Signs of estrus recorded included visual mounting activity and standing to be mounted (standing in estrus). Trained personnel from our laboratory administered all treatments of $PGF_{2\alpha}$ (0.5 mg cloprostenol; 2 mL of Estrumate, Merck Animal Health) and GnRH (100 µg gonadorelin diacetate tetrahydrate, 2 mL of Cystorelin, Merial) in

semimembranosus or semitendinosus muscles using single-dose 3 mL syringes with 20-gauge 3.5-cm needles. All procedures described in this manuscript were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University.

Pre-treatment

Heifers were pre-synchronized using $PGF_{2\alpha}$ followed in 2 d with GnRH to start a new cycle [21](Fig. 1). Only heifers that undergone complete CL regression in response to $PGF_{2\alpha}$ and ovulated to the GnRH were considered to start a new cycle at GnRH (d 0 of the cycle) and continued in the experiment. To determine if heifers had started a new cycle, ovaries of each heifer were scanned on d of GnRH and 2 d later with a MicroMaxx Sonosite ultrasound machine with a linear array transducer utilizing 10 MHz frequency (Sonosite Inc., Bothell, WA). All ovarian structures (follicles and corpora lutea) of each heifer were measured and recorded in an ovarian map immediately following GnRH administration. Largest transection size of follicles with antrum diameter ≥ 7 mm and corpora lutea (CL) were measured using build-in calipers. Follicles with antrum < 7 mm had their average diameter estimated using on-screen lateral grids. Ovulation was characterized by the disappearance of a dominant follicle (DF), followed by detection of a newly formed CL on the same ovary location of the DF 2 d after GnRH. Corpus luteum complete regression was determined by a decrease of the maximum luteal size and disappearance of luteal blood flow 4 d after $PGF_{2\alpha}$ injection [22,23]. The color function of the ultrasound machine was used to detect luteal blood flow.

Treatment

On d 6 of the estrous cycle, the first GnRH of Ovsynch (G1) was administered to induce a second follicular wave, and heifers were randomly assigned to four groups. Only heifers that

ovulated to G1 and start a new follicular wave were used in the experiment (n = 159). Ovulation to G1 was determined the same fashion as described for the pre-synchrony GnRH. Day of G1 was considered treatment d 0 and d 0 of the synchronized ovulatory follicular wave. The four groups (Figure 4.1) consisted of: high progesterone control (**HPC**; n = 33), low progesterone control (**LPC**; n = 45), and treatments **PG2** (n = 37) and **PG3** (n = 44). Heifers assigned to LPC, PG2 and PG3 received $PGF_{2\alpha}$ on treatment d 1 to regress all d 7 CL, which was formed after ovulation to pre-synchrony GnRH. Heifers from these treatments had 1 new CL growing during the treatment period that was formed after G1. Treatments PG2 and PG3 received another $PGF_{2\alpha}$ on treatment d 2 and 3, respectively. Heifers assigned to HPC did not receive $PGF_{2\alpha}$ on treatment d 1, thus had a mature and a new CL growing during the treatment period. On d 7 of treatment, $PGF_{2\alpha}$ of Ovsynch was administered in all heifers. A second $PGF_{2\alpha}$ was administered on d 8 to ensure complete luteal regression. The final GnRH of Ovsynch (G2) was administered on d 9. Ovulation to G2 was determined in a similar method as described previously with ultrasonography examination of ovaries immediately after the GnRH treatment and 2 d later. If a new CL was detected immediately after the last GnRH of Ovsynch, ovulation was determined to occurred prior to the last GnRH of Ovsynch.

Blood collection and progesterone assay

Blood sample was collected via coccygeal venipuncture on treatment d 7 (n = 157) and in a subset of heifers on treatment d 1, 2, 3, 4 (n = 82) to measure serum concentrations of progesterone by RIA (Coat-a-Count Progesterone, Siemens Diagnostic Solutions, Los Angeles, CA). After collection, samples were kept in a cooler with ice, transported to the laboratory and maintained in a refrigerator at 4 °C overnight. Serum was separated within 24 h after collection

by centrifugation at 2000 x g for 20 min at 4 °C and stored at -20 °C for later progesterone RIA. Intra- and inter-assay CV were 7.0 % and 8.7 %, respectively.

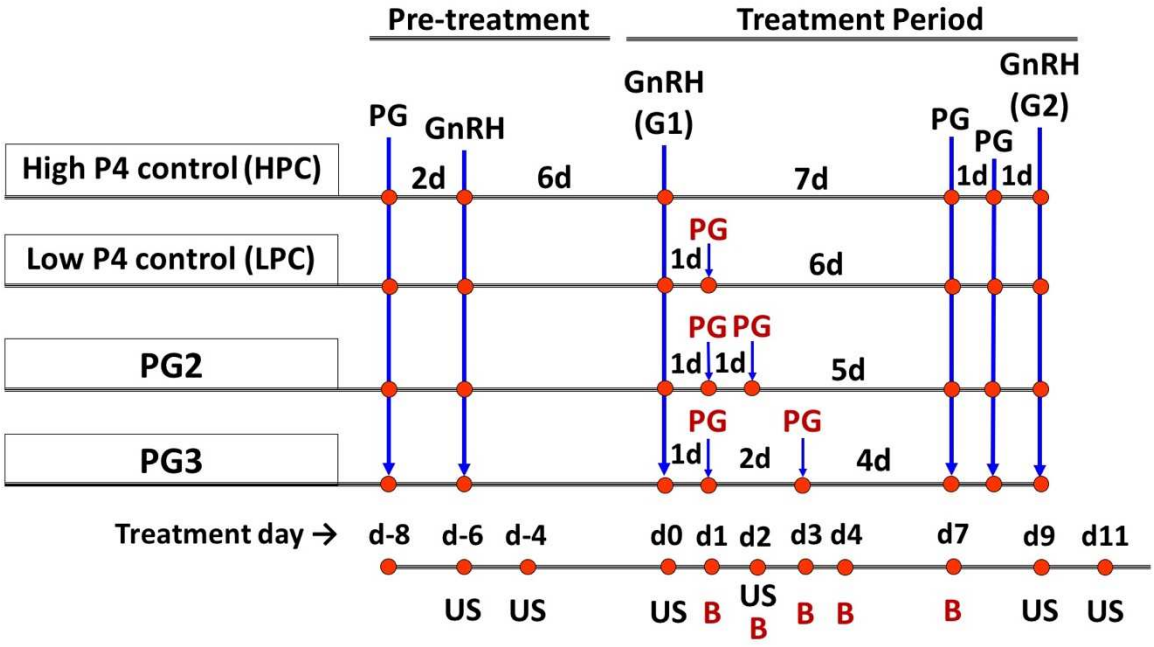

Figure 9.1. Schematic illustration of treatments, blood collections (B) and ultrasonography examinations (US). G1= first GnRH of Ovsynch; G2= final GnRH of Ovsynch; PG= $PGF_{2\alpha}$; US= Ultrasonography examination of ovaries; B= Blood sample collection.

Statistical analysis

Normal distribution of residuals was evaluated in each statistical analysis conducted in this study using Shapiro-Wilk test and residual plots. Normal distribution of residuals was not observed with a linear mixed model using the MIXED procedure with repeated measurements of SAS (version 9.4, SAS Inst. Inc., Cary, NC) when the effects of treatments on serum P4 levels was analyzed. Therefore, generalized linear mixed model using the GLIMMIX procedure with treatment day as a random effect and heifers nested in treatment specified in the SUBJECT option of SAS was used. The statistical model consisted of treatment, treatment day and the interaction treatment by treatment day.

Continuous variables such as size of pre-ovulatory follicle were analyzed using a linear mixed linear implemented with the MIXED procedure of SAS. Differences in variation of serum concentrations of P4 at time of $PGF_{2\alpha}$ of Ovsynch between treatment groups was also analyzed using the MIXED procedure followed by a CONTRAST statement. Treatment effect on percentage of heifers across different ranges of serum P4 at time of $PGF_{2\alpha}$ of Ovsynch, percentage of heifers detected in estrous and ovulatory response to the last GnRH of Ovsynch was analyzed by chi-square test using the FREQ procedure of SAS.

RESULTS

Pre-treatment validation

Mean diameter of pre-ovulatory follicles at time of pre-synchrony GnRH (P = 0.56; 14.0 ± 0.2 mm) and G1 (P = 0.53; 13.0 ± 0.1 mm) did not differ between treatments for heifers included in the study. Only four heifers (2/159) had double ovulation after pre-synchrony GnRH and 2 d-7 CL at time of G1. These four heifers were distributed in treatments HPC (n=1), LPC (n=1) and PG2 (n=2). One heifer (1/159) in treatment PG2 had double ovulation following G1 and 2 new CL growing during treatment period. Mean serum concentrations of P4 was not different (P = 0.46) between treatments on treatment d 1 (Figure 4.2; 3.89 ± 0.25, 3.67 ± 0.22, 3.64 ± 0.22 and 3.38 ± 0.20 ng/mL for HPC, LPC, PG2 and PG3, respectively), indicating that heifers of different treatments had similar luteal function prior to treatments and treatment effects were not confounded by pre-treatment.

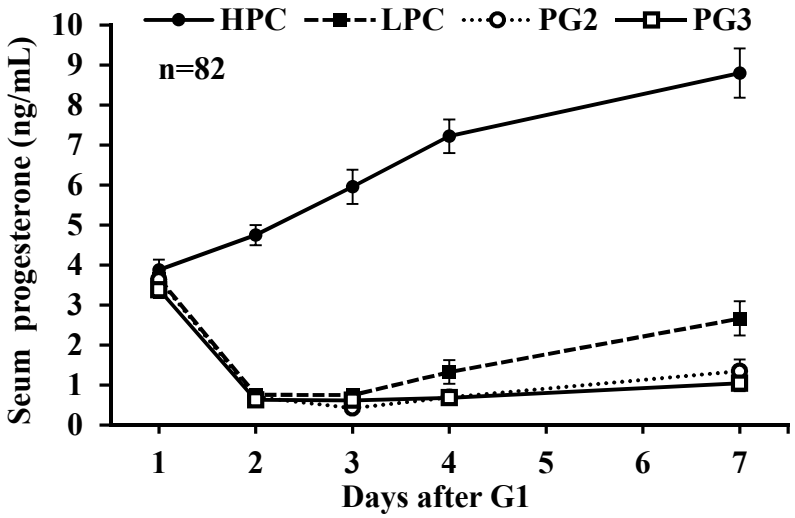

Figure 9.2. Mean ± SEM serum concentrations of progesterone (P4) from d 1 to 7 following the 1st GnRH of Ovsynch (G1; treatment d 0) in dairy heifers treated with HPC, LPC, PG2 and PG3. Only heifers that had blood samples collected on all these days were included in this figure (HPC, n = 19; LPC, n = 22; PG2, n = 18; PG3, n = 23). Mean serum of P4 did not differ between treatments on treatment d 1. HPC treated heifers had greater (P <0 .001) serum P4 compared to LPC, PG2 and PG3 on treatment d 2, 3, 4, and 7. On treatment d 7, LPC heifers had greater (P < 0.01) serum P4 compared to PG2 and PG3 heifers.

Effect of treatment on serum P4

Figure 4.2 shows the mean ± SEM serum concentrations of P4 for the four different treatments during the growth of the pre-ovulatory follicle for heifers with blood collection during the entire treatment period (n = 82). Administration of $PGF_{2\alpha}$ on treatment d 1 for heifers in groups LPC, PG2 and PG3 induced regression of the d-7 CL (formed after ovulation to pre-synchrony GnRH) based on the reduction of serum P4 to < 1.00 ng/mL on treatment d 2. Heifers with only a new CL growing (LPC, PG2 and PG3 treatments) during the ovulatory follicle development had lower mean serum concentrations of P4 between treatment d 2 to 7 compared to heifers with 2 CL, a mature and a new CL growing (HPC treatment). A single administration of $PGF_{2\alpha}$ on d of ovulation also impacted the development of the new CL formed after ovulation

since 27 % of heifers treated with LPC had serum P4 < 0.50 ng/mL at $PGF_{2\alpha}$ of Ovsynch (treatment d 7), and 18 % had serum P4 between 0.50 and 0.99 ng/mL (Table 4.1). Percentage of heifers with P4 < 1.00 ng/mL at time of $PGF_{2\alpha}$ of Ovsynch did not differ ($P \geq 0.40$) between treatments LPC (44.4 %), PG2 (40.5 %) and PG3 (50 %). However, mean serum concentrations of P4 at time of $PGF_{2\alpha}$ of Ovsynch was reduced for treatments PG2 and PG3 compared to LPC (Figure 4.2), indicating that a second administration of $PGF_{2\alpha}$ approximately 1 or 2 d after ovulation (PG2 or PG3, respectively) reduced the capacity of the new growing CL to produce P4.

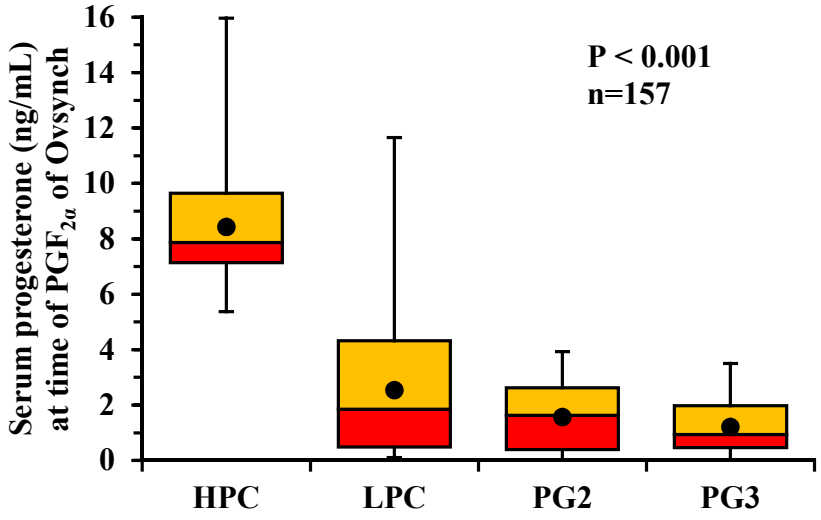

Figure 9.3. Box plot for serum concentrations of progesterone (ng/mL) at time of $PGF_{2\alpha}$ of Ovsynch (treatment d 7) for each treatment. All heifers that had blood samples collected on d of $PGF_{2\alpha}$ of Ovsynch were included (n = 157). The central box represents the inter quartile range (IQR) bounded by the first and third quartile (25th and 75th percentiles, respectively). Thus, 50% of the observed values lie within the range determined by the central box; the line inside the box represents the median or 50th percentile; the dot represents the mean. The whiskers are drawn between the quartiles and the corresponding extreme value of the group. A group having a larger box and whiskers is associated with a larger residual variance.

Variability in serum P4 on treatment d 7 was also reduced ($P < 0.02$) by a second $PGF_{2\alpha}$ administration 1 or 2 d after ovulation (PG2 and PG3, respectively; Figure 4.3) compared to just one $PGF_{2\alpha}$ administration at d of ovulation (LPC). Mean pre-ovulatory follicle diameter at G1 (P

= 0.56) and mean serum P4 1 d after G1 (P = 0.55) were not different between heifers with only a new CL in the 5 classes of serum P4 treatment d 7, which indicates that this two factors did not influence the CL responsiveness to $PGF_{2\alpha}$ treatment.

Table 9.1. Mean ± SEM serum concentrations of progesterone (P4) at time of $PGF_{2\alpha}$ of Ovsynch (treatment d 7) for treatments and percentage of heifers in classes of serum P4 concentrations at time of $PGF_{2\alpha}$ of Ovsynch within treatment. All heifers with blood samples at $PGF_{2\alpha}$ of Ovsynch were included in this table (n=157).

Treatment	n	Mean Serum P4 at $PGF_{2\alpha}$ of Ovsynch, ng/mL ± SEM	Serum P4 (ng/mL) ranges at $PGF_{2\alpha}$ of Ovsynch				
			< 0.50 % (n/n)	0.50 to 0.99 % (n/n)	1 to 1.99 % (n/n)	2 to 2.99 % (n/n)	≥ 3.00 % (n/n)
HPC	33	8.43^{a} ± 0.39	0^{a}	0^{a}	0^{a}	0^{a}	100^{a} (33/33)
LPC	45	2.55^{b} ± 0.36	27^{b} (12/45)	18^{b} (8/45)	7^{a} (3/45)	7^{ab} (3/45)	42^{b} (19/45)
PG2	37	1.58^{c} ± 0.20	32^{b} (12/37)	8^{ab} (3/37)	24^{b} (9/37)	22^{c} (8/37)	14^{c} (5/37)
PG3	42	1.21^{c} ± 0.15	26^{b} (11/42)	24^{b} (10/42)	26^{b} (11/42)	14^{bc} (6/42)	10^{c} (4/42)

^{a,b,c} Means within columns with different superscripts differ (P < 0.05).

Table 9.2. Percentage of heifers within treatment that were detected in standing in estrus on different days between $PGF_{2\alpha}$ of Ovsynch (treatment d 7) and 1 d after final GnRH of Ovsynch (G2; treatment d 10).

Heifers standing in estrus, % (n/n)	Treatments			
	HPC	LPC	PG2	PG3
1 and 2 d prior G2	0^{a} (0/33)	11^{b} (5/45)	14^{b} (5/37)	16^{b} (7/44)
On d of G2	6^{a} (2/33)	38^{b} (17/45)	43^{b} (16/37)	55^{b} (24/44)
1 d after G2	15^{a} (5/33)	4^{ab} (2/45)	0^{b} (0/37)	0^{b} (0/44)
Total, % (n/n)	21^{a} (7/33)	53^{b} (24/45)	57^{b} (21/37)	70^{b} (31/44)

^{a,b} Means within rows with different superscripts differ (P < 0.05).

Table 9.3. Mean ± SEM serum progesterone (P4) and percentage of heifers within treatment that ovulated after or prior the final GnRH of Ovsynch (G2), or that did not ovulate 2 d after G2. All heifers in the study were included in this table (n=159).

		Ovulatory response to the final GnRH of Ovsynch (G2)						
		Ovulation after G2			Ovulation prior to G2		No ovulation 2 d after G2	
Treatment	n	% (n/n)	Ovulatory follicle size, mm ± SEM	Mean serum P4 at $PGF_{2\alpha}$ of Ovsynch, ng/mL ± SEM	% (n/n)	Mean serum P4 at $PGF_{2\alpha}$ of Ovsynch, ng/mL ± SEM	% (n/n)	Serum P4 at $PGF_{2\alpha}$ of Ovsynch, ng/mL
HPC	33	100^{a} (33/33)	13.5^{a} ± 0.3	8.43^{a} ± 0.39	0^{a}	-	0^{a}	-
LPC	45	78^{b} (35/45)	15.8^{b} ± 0.4	3.06^{b} ± 0.41	20^{b} (9/45)	0.31^{a} ± 0.06	2^{a} (1/45)*	4.60
PG2	37	76^{b} (28/37)	15.6^{b} ± 0.5	1.99^{c} ± 0.21	22^{b} (8/37)	0.26^{a} ± 0.06	3^{a} (1/37)	0.70
PG3	44	77^{b} (34/44)	15.6^{b} ± 0.2	1.52^{c} ± 0.17	20^{b} (9/44)	0.25^{a} ± 0.06	2^{a} (1/44)	0.19

*Dominant follicle luteinized after G2.
^{a,b,c} Means within columns with different superscripts differ ($P < 0.05$).

Effect of treatment on estrus detection, ovulatory response to final GnRH of Ovsynch and pre-ovulatory follicle size

A greater ($P < 0.01$) percentage of heifers was detected standing in estrus between d of $PGF_{2\alpha}$ of Ovsynch (treatment d 7) and 1 d after the final GnRH of Ovsynch (treatment d 10) in treatments LPC, PG2 and PG3 compared to HPC (Table 4.2). There was also a shift on time of estrus with heifers in treatments LPC, PG2 and PG3 being detected in standing in estrus prior to the final GnRH of Ovsynch. A greater percentage of heifers in LPC, PG2 and PG3 had ovulation prior to the final GnRH of Ovsynch compared to heifers in HPC (Table 4.3). All heifers that ovulated prior to the final GnRH had serum P4 < 1.00 ng/mL at time of $PGF_{2\alpha}$ of Ovsynch. For heifers that ovulated following the G2, HPC heifers had a larger pre-ovulatory diameter

compared to LPC, PG2 and PG3 heifers (Table 3). Only 5 % (6/130) of total heifers that ovulated after G2 had double ovulation (n=3 in PG2, n=2 in LPC, and n=1 in PG3).

DISCUSSION

The central hypothesis of the present study was that a single im treatment of $PGF_{2\alpha}$ 1 or 2 d after ovulation would decrease serum P4 6 d after ovulation. Results indicated that heifers treated with $PGF_{2\alpha}$ 1 or 2 d after ovulation reduced mean serum concentrations of P4 compared to heifers with no treatment after ovulation. The current study was novel because it tested the effect of $PGF_{2\alpha}$ during the early stage of luteal development (d 1 or 2 after ovulation) in a new CL that was formed around d 7 of the estrous cycle. In contrast with our results, previous studies did not report a reduction in serum levels of P4 of heifers or cows using a single label dose of $PGF_{2\alpha}$ or its analogues administered im on d 2, 3 or 4 of the estrous cycle [4,12-14]. A reduction in circulating concentrations of P4 in cows or heifers was only reported after repeated treatments of $PGF_{2\alpha}$ after d 3 of the cycle [4,16-20], a double dose of $PGF_{2\alpha}$ after d 4 of the cycle [15,17,19], or a single dose on d 4.5 or 5 of the estrous cycle [13,18].

In fact, the majority of treated heifers in our study had serum P4 levels > 0.50 ng/mL, demonstrating that the young CL was resistant to complete luteolysis. However, ~ 29 % of treated heifers had serum P4 < 0.50 ng/mL 6 d after ovulation. These heifers were considered to undergone complete luteolysis. It was not known if the CL was going to recover after 7 d post ovulation since we administered $PGF_{2\alpha}$ of Ovsynch. A previous study also reported 2/9 (22 %) of cows with complete luteolysis following a double dose of $PGF_{2\alpha}$ on 3.5 d after ovulation [15]. Some heifers (27 %) in the LPC group also developed complete luteolysis 6 d after ovulation. We did not expect heifers treated with LPC undergoing complete luteolysis since all heifers in

the present experiment had serum levels of P4 > 1.60 ng/mL (mean ± SEM = 3.62 ± 0.11 ng/mL) on d 7 of the estrous cycle with just one d 7 CL. These heifers only received $PGF_{2\alpha}$ 1 d after first GnRH of Ovsynch to regress their d 7 CL. At this time, heifers could have a pre-ovulatory follicle or a newly formed corpus hemorrhagicum (CH) since ovulation occurs between 24 and 32 h after a GnRH-induced LH surge treatment [6]. In order to triggers luteolysis, $PGF_{2\alpha}$ binds to specific $PGF_{2\alpha}$ receptors (or FP receptors) that are located on membrane of steroidogenic large luteal cells [11,12,24,25]. Extremely low concentrations of FP receptors are present in granulosa or thecal cells on the pre-ovulatory follicle prior to ovulation [11], hence it is unlikely that $PGF_{2\alpha}$ directly affected the pre-ovulatory follicle. In the bovine CL, FP receptors appears to be formed during the luteinization process since in 1 and 2 d after ovulation, FP mRNA increased more than 500- and 2.500- fold, respectively, compared to pre-ovulatory follicles [11]. If heifers of LPC group already had ovulated at time of the $PGF_{2\alpha}$ 1 d after G1 just a small proportion (< 20%) of the FP receptors of a mature CL would be present in the CH [11]. Thus, it is not clear the mechanisms involved on the decrease of function of the newly formed CL with $PGF_{2\alpha}$ administration 1 d after GnRH of Ovsynch.

Studies identified different aspects of the $PGF_{2\alpha}$ response of the early CL (before d 5 of the estrous cycle) compared to the fully mature and responsive CL (after d 10). The lack of regression capacity of the newly formed CL does not appear to be due an absence of high affinity FP receptors or to their insufficient response to the $PGF_{2\alpha}$ stimulus [10-12]. However, some important intracellular pathway steps that were present in the responsive mature CL following administration of $PGF_{2\alpha}$ appears to be absent in the early CL [12]. There are several differences in gene expression between the two stages of CL development after administration of exogenous $PGF_{2\alpha}$ [26]. For instance, the early CL was unable to activate the intra-luteal $PGF_{2\alpha}$ synthesis

pathway [12] and the pathways involved in increasing intracellular calcium via calcium/calmodulin-dependent protein kinases (CAMKs) [26] that are vital for the process of luteolysis . In addition, angiogenesis-related genes were also differently expressed in the two stages of luteal development after $PGF_{2\alpha}$. Expression of the angiogenic gene fibroblast growth factor-2 (FGF-2) was increased in the d 4 CL after $PGF_{2\alpha}$ compared to the d 11 CL [27]. While antiagiogenic genes, inhibitors of the FGF-2 action, thrombospondin-1 and -2 , their receptor and pentraxin 3 were upregulated following $PGF_{2\alpha}$ administration only in d 11 CL [27]. Genes related to activation of the immune system might also not be affected by $PGF_{2\alpha}$ during early CL development. For example, a potent chemokine that attracts monocytes and macrophages, monocyte chemoattractant protein-1 (MCP-1), is upregulated by PG specifically in d 11 CL undergoing luteolysis . Thus, there are several factors that might be related to the ability of the early CL to resist complete luteolysis after $PGF_{2\alpha}$.

Different physiological state of particular individuals or peculiarities of our study experimental design might have influenced some heifers in our study to achieve complete luteolysis with an earlier CL. The advanced stage of the cycle that the new CL was formed might be a factor that influenced our results. In the current study, ovulation was induced with GnRH-induced LH surge (G1) on d 6 of the estrous cycle and a new CL was formed on d 7. The formation of this new CL in a different hormonal environment may have increased its susceptibility to $PGF_{2\alpha}$, potentiated the effects of $PGF_{2\alpha}$ or affected the development of the young CL. In addition, during the formation of this new CL, another mature d-7 CL was undergoing luteolysis. To our knowledge, it is not known if the presence of a mature CL (≥ 6 d after ovulation) undergoing luteolysis could affect the luteolysis efficiency of a newly formed CL. As previous discussed, the resistance of the early CL to $PGF_{2\alpha}$ administration appears to be

due to differences of the early CL to express genes important for the luteolytic process. The presence of a responsive mature CL undergoing luteolysis at time of early CL development might have contributed to the production of factors that were missing in the signaling pathway of the early CL. However, in order to test the effect of $PGF_{2\alpha}$ on early CL development in different stages of the estrous cycle or without an extra CL, our experiment would have to have a control group with a newly formed CL before d 5 of the estrous cycle.

Another objective of my thesis was to use $PGF_{2\alpha}$ during early CL development to create a model with reduced P4 levels during growth of the pre-ovulatory follicle. The goal was to use this model to determine the effect of low P4 before AI on fertility of dairy heifers. In order to create an optimal model, our treatment would have to reduce P4 to sub-luteal levels in heifers yet avoiding complete CL regression prior to $PGF_{2\alpha}$ of Ovsynch and ovulation of pre-ovulatory follicle prior to the final GnRH of Ovsynch. Duration of the ovulatory follicular wave of heifers in controls and treatments would have to be similar since it has an impact on fertility of cattle [28,29]. Unfortunately, present results showed that approximately 20 % of treated heifers had ovulation prior to the final GnRH of Ovsynch, indicating that time of ovulation was not properly synchronized after GnRH of Ovsynch. Heifers that ovulated prior to the GnRH of Ovsynch had P4 < 0.50 ng/mL prior to the final $PGF_{2\alpha}$ of Ovsynch. The increase of the percentage of cows detected on estrus prior to final GnRH of Ovsynch is another evidence that some heifers had a spontaneous LH surge prior to the final GnRH of Ovsynch. Therefore, to develop an optimal model that avoid ovulation of the ovulatory follicle prior to the final GnRH of Ovsynch, it might be necessary to use an exogenous source with low levels of P4 to keep heifers from undergoing complete luteolysis prior to the final $PGF_{2\alpha}$ of Ovsynch and ovulation prior to the final GnRH of Ovsynch.

CONCLUSIONS

$PGF_{2\alpha}$ treatment during early corpus luteum development reduced circulating P4 concentrations to sub-normal luteal levels compared with HPC and LPC. Treatments LPC, PG2 and PG3 were not able to maintain CL function 6 d after ovulation since approximately 29 % of heifers underwent complete CL regression prior to the $PGF_{2\alpha}$ of Ovsynch. In addition, approximately 20% of treated heifers ovulated prior to the final GnRH of Ovsynch. Although treatments with $PGF_{2\alpha}$ was able to reduce mean serum P4 in heifers, it did not effectively control CL function to be utilized as a model to test high vs. low serum P4 on fertility parameters.

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CHAPTER 10

REVIEW OF FINDINGS & RESEARCH AND PRACTICAL IMPLICATIONS

Reproductive efficiency of lactating dairy cows has major economic impacts on dairy operations. During the last 6 decades, P/AI of cows inseminated following estrus detection has decreased to alarming rates. Commercial dairy farms that only inseminate cows following estrus detection are susceptible to poor reproductive performance. In order to optimize fertility of lactating dairy cows and to reduce the dependency of estrus detection, timed-AI programs were developed. Currently, timed-AI programs have been used extensively in commercial dairy farms worldwide. Timed-AI programs demonstrated to increase P/AI compared to AI following estrus detection; however, there are still room for improvements on the logistics of the programs and on P/AI of lactating dairy following timed-AI.

The present dissertation was separated in two sections. The first section objectives were to: (1) compare currently most successful fertility treatments (timed-AI programs) in lactating dairy cows; (2) develop a simpler fertility treatment to be implemented in commercial dairy farms with logistical constrains; and (3) determine the relationship between progesterone during Ovsynch and fertility of lactating dairy cows. The overall hypothesis of the first section of this dissertation was that pharmacological control of ovarian development (timed-AI programs) that could increase percentage of cows with functional corpus luteum (CL) or serum concentrations of P4 during growth of the ovulatory follicle would maximize fertility of lactating dairy cows. The second section had the objective of determining the effect of high versus low serum concentrations of P4 during the growth of the ovulatory follicle on follicle development and fertility of lactating dairy cows and heifers. Our hypothesis was that low P4 would reduce fertility of lactating dairy cows by increasing percentage of pregnancy losses.

The study of Chapter 3 compared the currently most successful timed-AI programs in lactating dairy cows for first AI (Presynch-11, G6G and Double-Ovsynch-7d). The question of

our study was: will pre-synchrony strategy utilizing $PGF_{2\alpha}$ and GnRH enhance P/AI of lactating dairy cows compared to a pre-synchrony strategy using only $PGF_{2\alpha}$? Our findings indicated that even though pre-synchrony strategies that used $PGF_{2\alpha}$ and GnRH enhanced ovulation to the first GnRH of Ovsynch, P/AI was still similar between treatments. However, pre-synchrony strategies that combined $PGF_{2\alpha}$ with GnRH increased the percentage of cows with functional CL at the time of first GnRH of Ovsynch. Double-Ovsynch had a greater percentage of cows with functional CL at time of $PGF_{2\alpha}$ of Ovsynch compared to the G6G and Presynch-11. These results indicated that anovular cows have a better response to pre-synchrony programs that use $PGF_{2\alpha}$ and GnRH and support the use of Double-Ovsynch-7d for farms with a great percentage of anovular cows.

In this study, we also tested the reduction of the interval of the first GnRH of Ovsynch and the $PGF_{2\alpha}$ of the Ovsynch from 7 to 5 days. Even with the administration of two $PGF_{2\alpha}$ 8 h apart on the day of the $PGF_{2\alpha}$ of Ovsynch, the Double-Ovsynch-5d had a greater percentage of cows without complete luteolysis following $PGF_{2\alpha}$ of Ovsynch. This happened due to the refractoriness of the young CL to $PGF_{2\alpha}$ administration. Cows without complete CL regression after the $PGF_{2\alpha}$ of Ovsynch have almost no chance of conceiving after timed-AI. When only cows with CL regression were analyzed, Double-Ovsynch-5d had similar P/AI compared to the other treatments. Taken together, reduction of the interval of Ovsynch from 7 to 5 days impaired the fertility of lactating dairy cows. The use of this protocol in lactating dairy cows using only two $PGF_{2\alpha}$ 8 hours apart at time of $PGF_{2\alpha}$ of Ovsynch is not recommended.

One of the most promising data of this dissertation was the positive results achieved by the protocol PG+G presented in Chapters 4 and 5. PG+G program is a protocol that is simpler to be implemented on farms that have logistical constrains and reduced workforce availability

compared to fertility treatments such as G6G and Double-Ovsynch. Experiments in Chapters 4 and 5 compared different aspects of PG+G program with two other successful fertility treatments. Our question was: can administration of $PGF_{2\alpha}$ and GnRH simultaneously 7 d prior to Ovsynch be used as a pre-synchronization program? Surprisingly, PG+G program had similar P/AI to G6G and Presynch-10. Cows treated with PG+G had satisfactory percentage of cows with functional CL and serum concentrations of P4 at time of $PGF_{2\alpha}$ of Ovsynch. The PG+G program appears to have the potential to substitute other fertility programs. Although the results are promising, new research with larger number of cows are needed to include the PG+G protocol as a fertility program. Since the schedule for hormonal administration is very simple, PG+G can be implemented in farms that do not use fertility treatments due to logistical limitations or that only use estrus detection to inseminate cows.

In addition of using this program for first service, PG+G might be a great program to use for re-synchronization of cows diagnosed not-pregnant. Using PG+G as the only timed-AI program of lactating dairy for first or greater services would simplify reproductive management of the entire farm, reducing the risks of compliance issues and increasing reproductive performance of lactating dairy farms. The PG+G program can have massive implications on the dairy industry.

The studies present in this dissertation demonstrated that is important to utilize fertility programs that increase progesterone during growth of the ovulatory follicle, that increase the percentage of cows with ovulation to the first GnRH of Ovsynch, and ensure an extremely high percentage of cows with complete luteolysis following the PGF of Ovsynch. Presynch-11 or -10, G6G, Double-Ovsynch-7d, and PG+G demonstrated to be great timed-AI programs to be used in dairy farms. Before implementing this programs, dairy farmers should take in consideration their

management weekly schedule to avoid compliance issues. Cost and efficiency of the timed-AI program used on dairy farms should be evaluated before and after implementation of a timed-AI program.

A significant result from the study in Chapter 3 was the positive direct relationship between serum concentrations of P4 and P/AI of lactating dairy cows. Similar results have showed this correlation previously; however, our data strongly supported this finding with a large number of cows. Based on these findings and other data from the literature, we designed the study of Chapter 8. The question of our study was: Does reduced serum concentrations of P4 during the growth of the ovulatory follicle decrease pregnancy success after artificial insemination and increase pregnancy loss during gestation?

One of the most impressive results of this study was the precise manipulation of P4 achieved with the treatment groups. The treatments used achieved the expected concentrations of P4. Cows with high P4 had extremely high P4 compared to cows with physiological serum concentrations of P4 that are found in high producing lactating dairy cows. In the other hand, cows with low serum concentrations of P4 had extremely low P4 compared to serum concentrations that are also found in the literature for lactating dairy cows. Future research might use this model to identify mechanisms of selection of dominant follicle in cattle and to improve our understanding to the co-dominance phenomenon.

In Chapter 8, treatment with low circulating concentrations of P4 during growth of the ovulatory follicle increased the percentage of cows with double ovulations to 49 %, which is extremely high for bovine that is a monovular specie. Cows treated with low serum concentrations of P4 during the entire growth of the ovulatory follicle had also 4 times more double ovulations compared to cows treated with high P4 (12 %). Cows that had an event of

double ovulation during the pre-treatment had greater chance to have a double ovulation after treatment. This indicated that other factors, most likely genetic related, also impacted cows' susceptibility to double ovulations. Treatment with high P4 during the growth of the ovulatory follicle decrease the chance of the cows with history of double ovulation to have a double-ovulation after treatment, which might be a solution to decrease double ovulation in lactating dairy cows.

Results from experiment in Chapter 8 also demonstrated that double ovulation had a significant impact in fertility parameters of lactating dairy cows following AI. First, double ovulations increased P/AI at d 23 post-AI. Cows with double ovulations have two oocytes to be fertilized compared to just a single oocyte for cows with single ovulations. The increase on the number of oocytes ovulated appears to be the explanation to the increased percentage of cows pregnant at d 23 post-AI. A greater percentage of cows with double ovulations also had pregnancy losses between d 28 and 56 post-AI. Cows with unilateral double ovulations had greater percentage of losses compared to bilateral double ovulation cows and single ovulation cows, which indicated that most losses occurred due to unilateral twins during this period of gestation.

Based on the studies from this dissertation, it appears that low serum concentrations of P4 during the growth of the ovulatory follicle triggers a cascade of events starting with the higher incidence of double ovulations, which resulted in a greater percentage of unilateral twining, resulting in greater losses between d 28 and 56 of gestation. In Chapter 8, low P4 treatment also increased the percentage of cows with twin births, which is undesirable for lactating dairy cows due to the increased risk of problems during and/or following calving. For instance, twin births are related to increased incidence of dystocia and metabolic diseases. The increase in problems

during and/or following calving is also related to low fertility following calving. The increase in the percentage of problems caused by twin births make it undesirable for dairy producers who would probably treat cows to avoid twin births in their farms.

Chapter 8 indicated that the major impact of low P4 on pregnancy losses was through the increase on percentage of cows with double ovulation. However, it appears that low P4 might also have other mechanisms that increase percentage of pregnancy losses. A greater percentage of cows treated with low circulating concentrations of P4 during the ovulatory follicle dominance period that had single ovulation also had pregnancy losses between d 28 and 56 of gestation compared to cows treated with high P4. It would be important to develop new research to increase our understanding of the impact of low serum concentrations of P4 during the growth of the ovulatory follicle on single ovulating cows. The relatively high percentage of total pregnancy losses from days 23 to 265 d post-AI in all treatment groups demonstrated that pregnancy losses after d 23 of gestation has a major impact on fertility of lactating dairy cows. Research that increase our understanding of risk factors of pregnancy losses in cattle is fundamental to increase fertility of lactating dairy cows.

Results from Chapter 3 indicated a strong positive relationship between high serum P4 during Ovsynch. In this experiment, cows with high P4 (> 5 ng/mL) and luteolysis had P/AI at d 32 post-AI was approximately 55 %. However, in experiment of Chapter 8, cows treated with high P4 and single ovulation had 43 % for P/AI at d 35 post-AI. This result was lower than expected for the high P4 treatment. The reason for the reduced P/AI is not known but demonstrate that high circulating concentrations of P4 during ovulatory follicle development is not the only important factor in order to achieve high fertility of lactating dairy cows.

Results from the study of Chapter 9 indicated that dairy heifers were not affected by low circulating P4 as much as cows. In this experiment, only 6 % of heifers in low P4 groups had double ovulation comparing to 49 % of cows in the low P4 group of the experiment in Chapter 8. This demonstrated that during the transition of nulliparous heifer to lactating dairy cows several changes in the organism of the animal occur that impact reproductive physiology of high producing lactating dairy cows. Identifying these changes would be beneficial to understand the decrease in fertility that occur during the transition nulliparous heifer to lactating dairy cow.

The results from the experiments in this dissertation provided compelling evidence that hormonal environment during the growth of the ovulatory follicle have a significant impact on follicle dynamics and fertility of lactating dairy cows. However, it is clear that increasing serum concentrations of P4 during the growth of the ovulatory follicle is not the only solution to reduce pregnancy losses in lactating dairy cows. Determining key factors that impact ovulatory follicle development and contribute to pregnancy loss in lactating dairy cows is crucial to increase fertility of lactating dairy cows, which would lead to a preventive approach instead of therapeutic method to reduce pregnancy losses.