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 responses compared to when they were heifers that result in distinctly different reproductive measurements and pregnancy 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. Circulating concentrations of progesterone during diestrus are approximately half that in cows compared with 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 compared with 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 compared with heifers. Concentrations of progesterone during growth of the ovulatory follicle may be a key driver in differences in pregnancies per AI in cows compared with 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, Presynch-11, G6G and Double Ovsynch appear to enhance fertility of dairy cows. 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."

Keywords: corpus luteum, dairy, follicle, ovsynch, progesterone.

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 hormonal environment, production of oocytes and maintenance of pregnancy. The physiological outcomes of these structures in lactating dairy cows when compared with nulliparous heifers is associated with negative reproductive outcomes (Wiltbank *et al.*, 2006). Pharmacologic manipulation of ovarian structures can reverse these effects of lactation and enhance fertility (Wiltbank *et al.*, 2011). 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"; Sartori *et al.*, 2002, 2004; Wolfenson *et al.*, 2004). Differences in circulating concentrations of progesterone (P4) appears to be the key driver of many of these indices. Lactating cows have reduced circulating concentrations of P4 during the estrous cycle compared to heifers (Sartori *et al.*, 2002, 2004; Wolfenson *et al.*, 2004). This likely 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 compared with heifers (Bergfeld *et al.*, 1996). These differences in P4 and LH pulsatility also create differences in length of follicular waves (Wolfenson *et al.*, 2004). In this case, cows with lower concentrations of P4 have longer inter-wave intervals compared to heifers (Wolfenson *et al.*, 2004) 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 (Savio *et al.*, 1990). 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 (Wiltbank *et al.*, 2000, 2006; Sartori *et al.*, 2004; Lopez *et al.*, 2005). It appears the increase in twinning has a significant impact on pregnancy loss (López-Gatius *et al.*, 2002; López-Gatius and Hunter, 2005). Metabolic differences such as milk production and increased dry matter intake may explain these differences in reproductive function (Wiltbank *et al.*, 2006).

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 (Lucy, 2001; Washburn *et al.*, 2002). Current reports indicate that estrus detection rate

is ~36% in lactating cows 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 (Sartori *et al.*, 2004; Wolfenson *et al.*, 2004; Wiltbank *et al.*, 2006) as well as differences in environment, with cows spending more time on concrete (Platz *et al.*, 2008; Martin *et al.*, 2015) and being more susceptible to heat stress (Her *et al.*, 1988; Orihuela, 2000). Fortunately, estrus detection rate (or service rate when referring to cows timed-inseminated) is significantly enhanced when cows are timed-inseminated with Ovsynch (Pursley *et al.*, 1997a). 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 (Pursley *et al.*, 1997a, b). In the past 40 years, pregnancies per AI in lactating dairy cows decreased from around 65% in the 1950's (Spalding *et al.*, 1975; Butler and Smith, 1989) to approximately 35% (Strickland *et al.*, 2010; Fricke *et al.*, 2014) while pregnancies per AI in heifers remained steady at about 70% (Pursley *et al.*, 1997b; Escalante *et al.*, 2013). 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 (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006; Wiltbank and Pursley, 2014).

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-h 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 F2α (PGF2α) 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 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." (Wiltbank and Pursley, 2014) 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 (Pursley *et al.*, 1995). The first treatment, GnRH, may induce an LH surge and may cause a mature functional dominant follicle(s; DF) to ovulate (Sartori *et al.*, 2001). In turn, ovulation of the DF induces subsequent emergence of a new follicular wave ~1.5 days later (Pursley *et al.*, 1995) followed by development of a new dominant follicle during the next 7 days. If a follicle does not respond to the GnRH it is likely the cow is in the first 3 to 4 days of a follicular wave (Bello *et al.*, 2006). During this early stage of a wave, the largest growing follicle may be too immature (e.g., no LH receptors (Xu *et al.*, 1995) to respond to the GnRH-induced LH surge caused by the first GnRH treatment (Sartori *et al.*, 2001). 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 (Vasconcelos *et al.*, 2003). If the follicle becomes atretic prior to PGF2α, a new follicular wave will emerge. The new DF from that wave will most likely not be mature enough 2 days later to respond to the LH surge induced by the final GnRH of Ovsynch. If the PGF2α induces complete luteolysis, cows will likely display signs of estrus 3 to 4 days following AI following maturation of the newest pre-ovulatory follicle. This artifact is a common asynchrony of Ovsynch.

The second treatment, PGF2α, 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 PGF2α 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 PGF2α (Brusveen *et al.*, 2008) to induce a pre-ovulatory LH surge that triggers ovulation of the DF 24 to 32 h later (Pursley *et al.*, 1995). 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 *vs*. 39%, respectively; P > 0.10; Pursley *et al.*, 1997a). Up to 40% of cows may not have an ovulation synchronized with Ovsynch programs (Pursley *et al.*, 1995; Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). Non-synchronized cows will not be inseminated at an appropriate time relative to ovulation, or may not have luteolysis, thereby decreasing their chances of becoming pregnant (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). Improving synchronization rates alone of Ovsynch could have a dynamic impact on reproductive performance. Vasconcelos *et al.* (1999) 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 (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006).

Difference 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 PGF2α and GnRH compared to Ovsynch alone or

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Presynch-12 or -14/Ovsynch (Bello *et al.*, 2006; Souza *et al.*, 2008; Herlihy *et al.*, 2012). 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 day 6 or 7 of the new cycle (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). The data presented next will demonstrate that initiating the first GnRH of Ovsynch near day 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 (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). This, in turn, allowed more cows the opportunity for pregnancy, and translated into increased pregnancies per AI (Bello *et al.*, 2006).

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 (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). Ovulation to first GnRH of Ovsynch is followed by emergence of a new follicular wave, from which the ovulatory follicle of Ovsynch develops (Pursley *et al.*, 1995). 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 (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). This variation leads to a reduced chance of pregnancy (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006).

The G6G program, for example, decreased variability in size of the ovulatory follicle and increased synchronization rate to Ovsynch (Bello *et al.*, 2006). In these experiments, cows were treated with 25 mg PGF2α then 2 days later 100 µg GnRH. Then, cows received the first GnRH of Ovsynch either 4, 5, or 6 days later in experiment 1 (Bello *et al.*, 2006), and either 6, 7 or 8 days later in experiment 2 (Bello and Pursley, 2007). Controls in both experiments received only Ovsynch. Compared to Ovsynch alone, 6 days 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 PGF2α by luteolysis, and percentage of cows with both a luteolytic response to PGF2α and ovulation to the final GnRH of Ovsynch. These improvements were repeated in experiment 2 for day 6 compared to controls. In the two studies combined, ovulation rate to the first GnRH of Ovsynch averaged 90% in the day 6 groups (n = 76). In addition, it appears that 7 days 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, day 6 or 7 of the estrous cycle appear to be the ideal d of the estrous cycle to initiate Ovsynch to maximize ovulatory response to the first GnRH and

luteolysis following PGF2α (Bello *et al.*, 2006; Bello and Pursley, 2007).

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 PGF2α of Ovsynch, and a greater chance of ovulating to the final GnRH (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). Also from this study (Bello *et al.*, 2006), 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.

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 (Bello *et al.*, 2006; Bello and Pursley, 2007). In these preliminary data, we show nearly a doubling of percent cows pregnant in the 6 or 7 days groups compared to Ovsynch alone. Figure 1 describes some of the potential differences between two presynchronization schemes. Presynch utilizes two injections of PGF2α 14 days apart and 11 to 14 days prior to the start of Ovsynch. Since PGF2α 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 is approximately 61% in the 11 days interval, and approximately 45% in the 14 days interval, between second PGF2α and first GnRH of Ovsynch (Galvão *et al.*, 2007). In addition, cows treated with Ovsynch that are anovular will not respond to the PGF2α 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 day 6 of the cycle at time of first GnRH of Ovsynch, 97% of cows had accessory CL induced from the GnRH-induced LH surge, had significantly greater P4 concentrations, and had a greater probability of a pregnancy (Bello *et al.*, 2006; Bello and Pursley, 2007). Cows with both a day 7 and 13 corpora lutea at time of PGF2α of Ovsynch have approximately 50% greater P4 concentrations compared to cows with only a day 13 corpus luteum (Pursley and Martins, 2011). 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 P4 at time of induced luteolysis, and a greater chance for pregnancy.
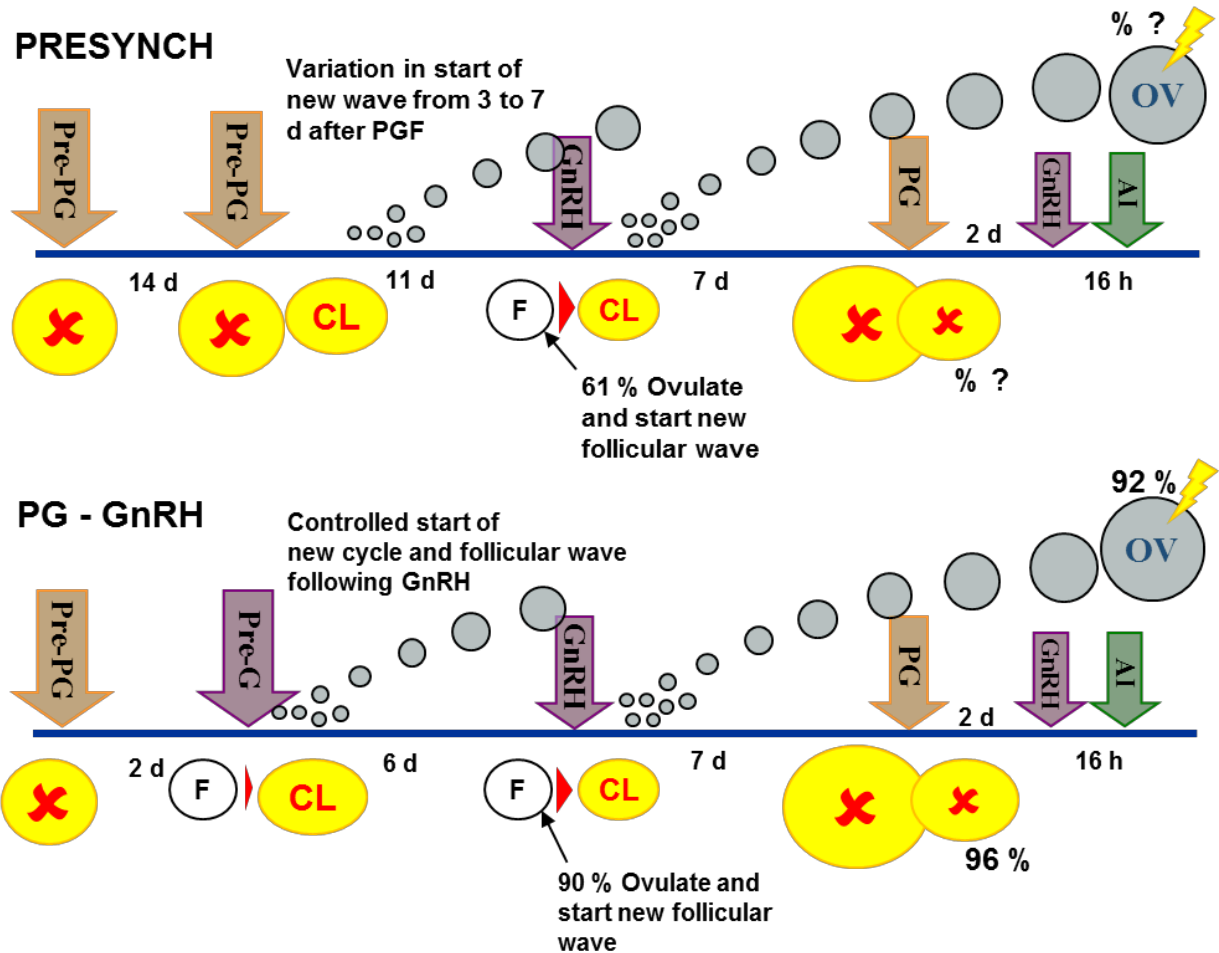
Figure 1. Description of potential difference in control of follicle development in Presynch versus the PGF2α – GnRH presynchronization scheme proposed. Control of a new estrous cycle in more cows may allow the PGF2α – GnRH scheme greater control of the ovulatory follicle following the first GnRH of Ovsynch. PG: PGF2α; F: Dominant follicle; AI: artificial insemination.

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 PGF2α on fertility of lactating dairy cows previously discussed; serum concentrations of P4 following PGF2α of Ovsynch has also been associated with P/AI of lactating dairy cows after timed-AI (Souza et al., 2007; Brusveen et al., 2009; Martins et al., 2011a). 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 (Souza et al., 2007; Brusveen et al., 2009; Martins et al., 2011a). Time to reach complete luteolysis after PGF2α of Ovsynch appears to also influence fertility since cows with a delay on P4 clearance had impaired fertility following timed-AI (Martins et al., 2011a, b). Therefore, it is essential that cows with functional CL

have complete luteal regression following PGF2α 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 PGF2α of Ovsynch are between 5 and 30% (Souza et al., 2007; Brusveen et al., 2009; Bisinotto et al., 2010; Martins et al., 2011a; Giordano et al., 2012). In addition, similar results were obtained using either of the two PGF2α 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 day 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 PGF2α of Ovsynch. A previous study from our laboratory identified that parity and service could affect percentage of cows with complete luteolysis (Martins et al., 2011a). 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%; Martins et al., 2011a). This same study also reported that cows with greater circulating of

P4 at time of PGF2α of Ovsynch had a greater probability of complete luteal regression (Fig. 2; Martins *et al.*, 2011a). This result was unexpected since cows with two CL, a mature and accessory CL, at time of PGF2α of Ovsynch have higher serum P4 at time of PGF2α of Ovsynch and were believed to have problems with luteolysis due the number of CL and the young age of the accessory CL (day 7).

In order to enhance percentage of cows with complete luteolysis following PGF2α of Ovsynch, two different approaches have been tested: repeated administrations with PGF2α commercial label dose (Brusveen *et al.*, 2009) and increased label dose of PGF2α administered once (Giordano *et al.*, 2013). Percentage of cows with complete CL regression was increased when an additional PGF2α treatment was administered 24 h after the PGF2α of Ovsynch (95.6%) compared to cows with only the PGF2α of Ovsynch (84.6%; Brusveen *et al.*, 2009). There was an increase in percentage of cows with luteolysis. P/AI in cows with two *vs.* one PGF2α treatment was 52.7 *vs.* 47.0% (Brusveen *et al.*, 2009). Based on data from Martins *et al.* (2011a), cows that do not have complete luteolysis have nearly a 0% chance of becoming pregnant. The 11% of cows that did not have complete luteolysis (95.6 - 84.6%) likely did not become pregnant and mathematically this calculates to nearly a 6% difference.

Giordano *et al.* (2013) tested if a 50% increase in the label dose (0.5 mg *vs.* 0.75 mg) of a PGF2α analogue (cloprostenol) would increase the percentage of cows with complete luteolysis following PGF2α of Ovsynch (Giordano *et al.*, 2013). The greater dose of PGF2α 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%; Giordano *et al.*, 2013). However, it did not influence primiparous cows (92.8 *vs.* 89.7%; Giordano *et al.*, 2013). These studies indicated that some cows and/or their CL are more resistant to achieve complete luteolysis with the regular label dose of PGF2α. Taken together, there is compelling evidence indicating that insufficient luteolysis after PGF2α of Ovsynch has a direct impact on reproductive performance of lactating dairy cow and that two administrations of PGF2α 24 h apart can overcome this problem. Therefore, fertility treatments incorporated the use of second PGF2α treatment 8 to 24 h after the PGF2α of Ovsynch to enhance complete luteolysis rate and the chances of pregnancy following timed-AI.
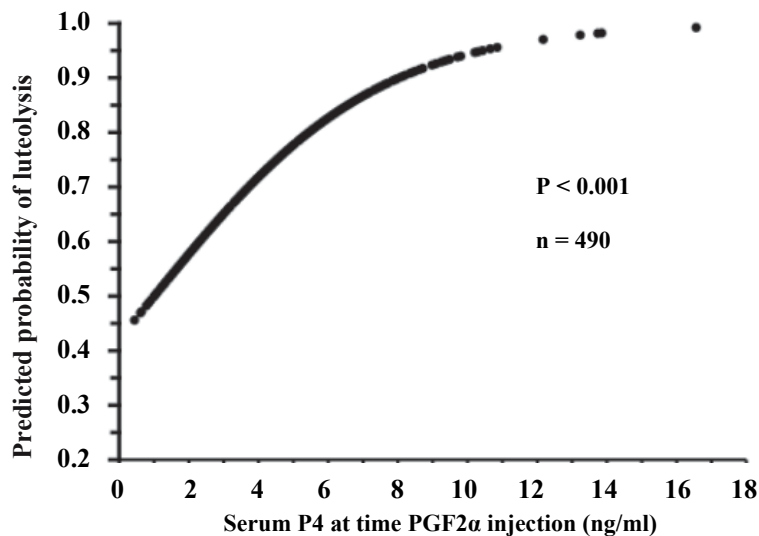

Figure 2. Predicted probability of complete luteolysis based on concentrations of progesterone (P4 < 0.5 ng/ml 56, 72, and 96 h after PGF2α injection) at time of PGF2α injection of Ovsynch in lactating dairy cows with functional corpus luteum (CL; P4 concentrations ≥0.24 ng/ml 24 h and ≥0.09 ng/ml 56 h after treatment; n = 490) at time of treatment. Published in Journal of Dairy Science (Martins *et al.*, 2011a).

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 day 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 PGF2α 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 (Sartori *et al.*, 2002, 2004; Wolfenson *et al.*, 2004). 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% (Bello *et al.*, 2006; Galvão *et al.*, 2007;

Souza *et al.*, 2008; Ribeiro *et al.*, 2012; Astiz and Fargas, 2013; Dirandeh *et al.*, 2015).

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