OPTIMIZING REPRODUCTIVE EFFICIENCY IN PREVIOUSLY INSEMINATED LACTATING DAIRY COWS AND PHYSIOLOGICAL RESPONSE TO INTRAVAGINAL DELIVERY OF GNRH AND PGF2α

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ABSTRACT

The objectives of the experiments presented in Section I were: to determine embryo mortality (EM) around the period of maternal recognition of pregnancy and identify alterations in ovarian dynamics in cows that suffered EM, and evaluate resynchronization programs based on ovarian structures present at nonpregnancy diagnosis (NPD) to maximize reproductive efficiency of lactating dairy cows. The objectives of the experiments presented in Section II were to test the feasibility of inducing luteal regression and an LH surge after intravaginal (IVG) administration of PGF2α and GnRH, respectively.

The first experiment of Section I demonstrated that combining data for mRNA expression of interferon stimulated genes in white blood cells and circulating concentration of pregnancy specific protein B is a useful method to identify cows undergoing EM. In addition, we observed that only cows undergoing EM after Day 24 of gestation had extended luteal phases and EM did not cause major alterations to the follicular wave dynamics after the onset of luteolysis. The other experiments presented in Section I demonstrated that a resynchronization of ovulation program (Short-Resynch) without the first GnRH treatment 25 ± 3 days after AI and in which cows were differentially treated based on the ovarian structures present at NPD (32 ± 3 days after AI) resulted in a greater proportion of cows re-inseminated at estrus without negatively affecting overall pregnancy per AI when compared with the Ovsynch protocol initiated 25 ± 3 days after

AI. In another experiment, Short-Resynch increased hazard of pregnancy after first service when compared to blanket use of the Ovsynch protocol initiated 32 ± 3 days after AI.

Experiments presented in Section II demonstrated that two IVG treatments of PGF2α 12 hours apart caused a decline in progesterone concentration similar to that observed after a single intramuscular luteolytic dose of PGF2α. Further, we observed that IVG instillation of GnRH analogues in combination with citric acid as an absorption enhancer elicited an LH surge of similar magnitude than after intramuscular administration of GnRH.

BIOGRAPHICAL SKETCH

Robert Wijma was born on February 25, 1987 in Montevideo, Uruguay. He graduated with a doctorate in veterinary science from the Universidad de la República (Uruguay) in 2011, and received the academic excellence award for the highest GPA in his class. He worked as a research intern in in vitro fertilization in sheep at IRAUy foundation (Uruguay) from 2010 to 2011. From 2012 to 2013 he worked as a research fellow at the dairy unit of the national institute for agricultural research of Uruguay. In 2013 he joined the PhD program at Cornell University in the Department of Animal Science, under the supervision of Dr. Julio O. Giordano.

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SECTION I

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CHAPTER I

ESTROUS CYCLE IN CATTLE AND SYNCHRONIZATION OF OVULATION IN LACTATING DAIRY COWS

1. General Introduction

Optimizing reproductive efficiency of dairy cattle is paramount to maximize dairy herd profitability (De Vries, 2006; Giordano et al., 2011; Giordano et al., 2012c). Optimizing time to pregnancy during lactation maximizes the time cows spend at stages of their lactation at which income over feed cost is maximized during their life time. In addition, cows that conceive early once eligible to become pregnant are less likely to be removed from the herd (Pinedo et al., 2010), provide replacement animals to maintain or grow the herd, and require fewer reproductive treatments and inseminations. Thus, reproductive success of dairy farms is determined by a combination of factors inherent to cow health, nutrition, management, and economic conditions. A healthy transition into lactation allows rapid uterine involution and resumption of ovarian cyclicity. Thereafter, reproductive success is determined by the rate at which cows are inseminated and the success of these inseminations. Therefore, an ideal reproductive management program for dairy cattle: (1) minimizes the interval from the end of the voluntary waiting period to the first artificial insemination (AI), (2) minimizes the interval between two

subsequent inseminations in cows that fail to conceive, and (3) maximizes fertility of all AI services (Giordano et al., 2013).

In the last decades, a better understanding of the physiological processes governing reproductive events in lactating dairy cows led to the development of synchronization of estrus and ovulation programs. These programs allowed the dairy industry to dramatically improve both insemination and conception risk in dairy herds (Wiltbank and Pursley, 2014). Further, the physiological responses to synchronization of ovulation protocols for first AI postpartum have been recently optimized leading to major gains in fertility (Moreira et al., 2001; Bello et al., 2006; Souza et al., 2008). Conversely, optimization of synchronization programs for second and greater AI has been more difficult. In part, this is because most resynchronization protocols have been developed based on assumptions about the reproductive physiology of non-inseminated cattle when in fact, they are used to synchronize ovulation in previously inseminated nonpregnant cows. These cows may present different ovarian dynamics than non-inseminated cows. For example, it is well known that embryo mortality during the period of embryonic development (i.e., up to 42 d after insemination) is prevalent in lactating dairy cows. During this period, the embryo produces and secretes factors into the uterine lumen to prevent luteal regression and therefore puts a transitory end to cyclicity, which needs to resume after embryo demise. In this regard, the effect of embryo mortality on ovarian dynamics and the response to resynchronization of ovulation programs is poorly understood.

This chapter aims to describe basic principles of estrous cycle physiology in cattle, current knowledge about embryo mortality in cattle, and synchronization of ovulation programs for lactating dairy cows.

2. Estrous cycle in cattle

2.1. Hormonal regulation

The estrous cycle is the combination of physiological, histological, and behavioral changes that take place from one estrus event to the next. These cyclic changes are controlled by finely tuned interactions among the main hormones and factors released by the hypothalamus, pituitary gland, ovaries, and uterus (Goodman and Inskeep, 2006). The estrous cycle can be divided into four stages: proestrus, estrus, metestrus, and diestrus. The endocrine environment during the first two is characterized by high concentration of estradiol (E2) produced by the pre-ovulatory follicle and low concentration of progesterone (P4), whereas P4 produced by the corpus luteum (CL) is the predominant ovarian steroid during metestrus and diestrus.

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are critical for the regulation of the estrous cycle. These hormones are synthesized and secreted by the gonadotropic cells of the pituitary gland in response to gonadotropin releasing hormone (GnRH) produced by the hypothalamus (Crowe, 1999). The latter is secreted in pulses and in response to each pulse, a pulse of LH is released (Welt et al., 2002). On the other hand, the predominant pattern of FSH secretion is not pulsatile but rather basal and mostly regulated by circulating concentrations of E2 and inhibin rather than by GnRH pattern of secretion (Padmanabhan et al., 2002). Although P4 secretion by the CL in cattle is mostly constitutive, LH may also play a role in normal development and function of the CL (Niswender et al., 2000). During the different phases of the estrous cycle E2 and P4 modulate GnRH and gonadotropin secretion through a complex feedback loop system which involves different neuronal nuclei and neuropeptides.

During proestrus and estrus, when P4 concentration is low, E2 produced by the dominant follicle establishes a positive feedback on LH. As a result, LH secreted in a small amplitude and

high frequency pulse pattern. This positive feedback loop concludes with an E2 surge which induces estrous behavior and triggers a surge of LH which, in turn, induces ovulation. Conversely, due to the elevated circulating concentration of P4 during diestrus, E2 and P4 combined reduce LH pulsatility which results in high amplitude and low frequency pulses of LH (Goodman and Inskeep, 2006). Follicle stimulating hormone secretion is downregulated by E2 and Inhibin secreted by the dominant follicle. After ovulation or atresia of the dominant follicle, E2 and inhibin concentrations decrease due to disappearance of the dominant follicle allowing FSH circulating concentration to rise and stimulate the beginning of a new follicular wave within 24 to 36 hours of ovulation (Goodman and Inskeep, 2006). On the other hand, luteal regression is regulated by prostaglandin F2α (PGF2α) secreted by the endometrium and oxytocin secreted by the anterior pituitary gland and the CL itself (Mccracken et al., 1999). Luteolysis is discussed in detail later in this chapter.

2.2. Follicular dynamics

Follicular development occurs during the estrous cycle in cattle and is characterized by a wave like pattern of growth and regression of follicles (Rajakoski, 1960). The introduction of transrectal ultrasonography (TUS) to monitor growth and regression of individual antral follicles allowed the validation of the initial hypothesis of a wave-like pattern of growth and regression of ovarian follicles (Sirois and Fortune, 1988; Ginther et al., 1996). The number of follicular waves per cycle varies with a predominance of two waves per cycle in lactating Holstein cows and predominance of three waves per cycle in non-lactating heifers (Sartori et al., 2004). The first follicular wave emerges 1 to 2 days after ovulation (Day = 0). In two wave cycles, emergence of the second wave occurs around Day 9 or 10. In three wave cycles, the second wave emerges

around Day 8 or 9 and the third wave around Day 15 or 16 (Adams et al., 2008). Each wave of follicular growth can be divided into three phases: recruitment, selection, and dominance. Recruitment is characterized by escape from undergoing atresia of a group of small antral follicles (3 to 4 mm) in response to an increase in FSH concentration (Fortune, 1994). After emergence, follicles grow at a similar rate for approximately two days until the time of selection, which is defined as the time at which the future dominant follicle reaches the maximum difference in growth rate with the largest subordinate follicle. At this point, the future dominant follicle is approximately 6.5 to 8 mm in diameter (Ginther et al., 1996). The ensuing period of dominance is characterized by continuous growth of the dominant follicle at a higher rate than subordinate follicles until ovulation or atresia (Ginther et al., 1996). In lactating dairy cows, the dominant follicle of a non-ovulatory wave usually grows until it reaches approximately 15 to 17 mm in diameter. Average size of the ovulatory follicle in lactating cows is 16 to 18 mm (Sartori et al., 2004). After ovulation or atresia of the dominant follicle, the circulating concentration of E2 decreases dramatically allowing the occurrence of a surge of FSH, and thereby the emergence of a new follicular wave (Ginther et al., 1996).

At approximately the time of deviation in follicle growth, FSH secretion is suppressed by E2 and inhibin produced predominantly by the dominant follicle. Because subordinate follicles are still FSH dependent, lower circulating levels of FSH result in follicle regression and atresia (Aerts and Bols, 2010). In contrast, granulosa cells of the dominant follicle express LH receptors allowing continuous growth in a low FSH environment (Ginther et al., 1996). Biochemical differences between dominant and subordinate follicles arise as early as the recruitment stage. For example, it has been documented that the dominant follicle has lower abundance of IGF-1

binding proteins in the follicular fluid, thus greater abundance of free IGF-1 has been proposed as the cause of greater growth rate (Fortune et al., 2004).

During follicular waves in which a dominant follicle develops to pre-ovulatory size in the presence of elevated P4 concentrations and increased E2 secretion, the hormonal environment prevents the occurrence of an LH surge thereby preventing ovulation. In addition, reduced LH pulsatility will lead to atresia of the dominant follicle and emergence of a new follicular wave due to lack of inhibition of FSH release (Ireland et al., 2000). Conversely, the positive feedback loop on the hypothalamus and pituitary generated by the combination of reduced circulating P4 and elevated E2 concentrations in circulation after CL regression leads to increasing GnRH and LH pulsatility, which will ultimately cause a surge of LH triggering ovulation and the formation of a new CL (Jeong and Kaiser, 2006).

2.3. Corpus luteum formation, growth, and regression

The CL is a transitory gland formed from granulosa and theca cells of the ovulatory follicle. The main function of this gland is to secrete P4. After ovulation, cells from the theca and granulosa of the ovulatory follicle reorganize to form the CL which reaches it maximum volume approximately 10 to 14 days after ovulation (Sartori et al., 2004). Progesterone concentration increases gradually after ovulation reaching 1 ng/mL approximately three to four days after ovulation and maximum levels (~7 ng/mL in heifers and 5 ng/mL in lactating dairy cows) around 12 to 14 days after ovulation (Sartori et al., 2004).

In the absence of pregnancy luteal regression is usually triggered ~16 to 19 days after the previous ovulation. The mechanism of luteolysis is governed by finely tuned interactions among the pituitary gland, ovaries, and uterus which will ultimately derive in endometrial (luminal and

glandular epithelium) secretion of the luteolysin PGF2α (Mccracken et al., 1999). Prostaglandin F2α secretion in the endometrium is stimulated by oxytocin [OT; (Roberts et al., 1976)] which is secreted in a pulsatile manner by the neurohypophysis, and by the CL in response to PGF2α. Whether PGF2α secretion is triggered by OT coming from the neurohypophysis or from the CL is still a matter of controversy. Regardless, once OT stimulates PGF2α secretion in the endometrium, each pulse of PGF2α is usually followed by an OT pulse from the CL establishing a positive feedback loop (Flint et al., 1990). Four to six pulses of PGF2α within 24 hours are necessary to effectively induce complete CL regression. Progesterone concentration drops below 1 ng/mL within 24 to 48 hours after the onset of luteolysis (Ginther et al., 2009). It has been hypothesized that the pulsatile secretion pattern of PGF2α is required because prolonged exposure to elevated concentrations of PGF2α desensitizes luteal cells to PGF2α making them refractory to the luteolytic signal (Skarzynski and Okuda, 1999).

Almost 99% of the PGF2α that reaches the systemic circulation is metabolized during first passage through the lungs (Davis et al., 1980). Thus, to overcome the limitation of PGF2α reaching the ovary through the systemic circulation, ruminants developed a specialized counter current transport mechanism of PGF2α from the uterine vein to the ovarian artery through the utero-ovarian plexus (Lee et al., 2010). This mechanism is mediated by a transmembrane transporter located in the three layers (tunica intima, tunica media, and tunica adventitia) of the uterine vein and ovarian artery which allows PGF2α to reach the ovary by diffusion from the uterine vein into the ovarian artery (Lee et al., 2010).

The process of luteal regression can be divided into functional luteolysis (cessation of P4 synthesis and secretion), and structural luteolysis [loss of cells that form the CL structure; (Niswender et al., 2000)]. The immediate decrease in circulating P4 concentration after exposure

to pulses of PGF2α has been attributed to a reduction in luteal blood flow, mainly in response to an increase in the levels of the potent vasoconstrictor endothelin-1 (Niswender et al., 2000). Further, during luteolysis P4 secretion is reduced due to downregulation of steroidogenic acute regulatory protein [StAR; (Tsai et al., 2001)]. Downregulation of StAR limits cholesterol transport to the inner mitochondrial membrane, which is the first rate limiting step in steroid hormone synthesis (Stocco and Clark, 1996). On the other hand, structural regression is mediated through apoptosis and immune infiltration (Brännström and Norman, 1993).

The CL contributes to the regulation of its own lifespan because P4 produced by the CL regulates endometrial expression of the P4 receptor (PR), E2 receptor (ER), and oxytocin receptor (OXTR). After ovulation and CL formation, exposure to P4 progressively downregulates OXTR and ER, in the endometrium (Mccracken et al., 1999). Thus, during the first 5 to 7 days after ovulation ER (Kimmins and Maclaren, 2001; Robinson et al., 2001) and OXTR (Wathes and Hamon, 1993; Mann and Lamming, 1994; Robinson et al., 2001) are downregulated in the endometrial luminal epithelium and superficial glandular epithelium, preventing the pulsatile secretion of PGF2α. After 15 to 18 days of exposure to P4, the endometrium becomes refractory to P4 through downregulation of PR (Boos et al., 1996; Kimmins and Maclaren, 2001; Robinson et al., 2001). As a result, OXTR is upregulated in the endometrium (Wathes and Hamon, 1993; Mann and Lamming, 1994; Robinson et al., 2001) facilitating the luteolytic mechanism. Further, upregulation of OXTR mediated by E2 has been demonstrated in sheep (Wathes and Lamming, 1994). In cattle, E2 appears to have a positive effect on OXTR expression; however, it remains to be determined if E2 stimulation is necessary for OXTR upregulation (Leung and Wathes, 2000).

3. Maternal recognition of pregnancy

If conception takes place, the luteolytic process must be abrogated to maintain pregnancy. In this regard, the process by which the embryo signals the cow to prevent luteolysis is known as maternal recognition of pregnancy (MRP). In cattle, the embryo reaches the uterus by approximately Day 4 to 6 after ovulation and hatches from the zona pellucida by Day 9 to 10. Thereafter the conceptus begins to elongate growing from ~2 mm on Day 12 to approximately 20 cm by Day 19 (Hue et al., 2007). Beginning approximately on Day 12 to 14 after ovulation, mononuclear cells of the trophoblast portion of the elongating conceptus start to produce and secrete substantial amounts of interferon-τ [IFNT; (Farin et al., 1990)]. Interferon-τ is a type I interferon, homologous to IFN-α and IFN-ß with secretion limited to the placenta of ungulates (Roberts et al., 2008). Interferon-τ is believed to be the first signal to abrogate luteolysis and maintain pregnancy acting in a paracrine manner on the endometrium of the gravid uterine horn (Lamming et al., 1995). The ability of IFNT to prevent luteolysis has been demonstrated in several studies where intrauterine administration of IFNT extended luteal lifespan in cattle (Knickerbocker et al., 1986; Meyer et al., 1995) and sheep (Godkin et al., 1984; Ott et al., 1993).

The proposed mechanism by which IFNT seems to mediate maternal recognition of pregnancy is through down regulation of OXTR expression in the luminal and glandular epithelium of the endometrium. In sheep, it has been shown that IFNT exerts its effect on OXTR expression through downregulation of the ER (Spencer et al., 1995; Bazer et al., 1997). Conversely, there is controversial data regarding the role of E2 and ER in the regulation of the luteolytic mechanism in cattle. Some experiments have shown that ER remains unchanged during early pregnancy and therefore may not have a role in the down regulation of OXTR (Robinson et al., 1999), whereas others have shown downregulation of the ER on Days 16 and

18 of pregnancy (Robinson et al., 2001). More recently, Araujo et al. (2009) showed that maintaining low concentration of circulating E2 through follicular ablation delayed the onset of luteolysis in heifers, suggesting that E2 plays a role in luteolysis in cattle. Nonetheless, luteolysis could not be prevented through prolonged ablation either because E2 concentration was not reduced sufficiently or because E2 is not completely necessary in the luteolytic cascade.

By the time the endometrium desensitizes to P4 due to loss of PR, IFNT acting through a paracrine manner prevents the expression of OXTR and as a consequence pulsatile release of PGF2α thus, preventing the luteolytic cascade. The exact duration of the period of substantial IFNT secretion in cattle has not been clearly determined. Whereas it has been documented that substantial IFNT secretion begins around Day 16 of pregnancy (Mann and Lamming, 2001; Robinson et al., 2006), there is limited research regarding the end of IFNT secretion. Godkin et al. (1988) and Geisert et al. (1988) suggested that IFNT secretion can continue for as long as Day 30 of pregnancy, however, assays used in these experiments could not discriminate between IFNT and other similar conceptus secreted proteins. Most experiments documented greatest IFNT secretion from 16 to 19 d after conception (Robinson et al., 2006; Forde et al., 2014). Although there is evidence that IFNT reaches the systemic circulation (Han et al., 2006; Gifford et al., 2007; Green et al., 2010), a limitation to study the pattern of IFNT secretion dynamics in vivo is that circulating concentrations are very low which has limited assay sensitivity. To the best of my knowledge there is not an accurate method to measure circulating concentration of IFNT at the moment.

In summary, most research studies have shown greatest secretion of IFNT from ~14 to 19 days after conception. To the best of my knowledge there is not conclusive data regarding the duration of IFNT secretion and the extent of exposure to IFNT required to prevent luteolysis or

aid in pregnancy maintenance. In addition, the exact process or molecules involved in preventing luteal regression after the initial period of CL maintenance through effects mediated by IFNT remains unknown.

4. Embryonic mortality in lactating dairy cows

4.1. Fertilization rates in lactating dairy cows

Pregnancy wastage due to embryo mortality in dairy cattle has been recognized as a major cause of economic losses (De Vries, 2006; Diskin and Morris, 2008) and has been subject of study for more than 40 years (Ayalon, 1978). Embryo mortality refers to death of the embryo between fertilization and the completion of organogenesis, which occurs approximately 42 days after conception in cattle (Hubbert, 1972). Traditionally, early embryonic mortality referred to those occurring before the initiation of maternal recognition of pregnancy (~ Days 14 to 19 after AI), whereas late embryonic mortality referred to those occurring between the time of MRP and Day 42 of pregnancy (Humblot, 2001; Whitlock and Maxwell, 2008). Recent experiments have shown that fertilization rates in lactating dairy cows range from 55 to 85% depending on lactation status and season of insemination (Sartori et al., 2002; Cerri et al., 2009a; Cerri et al., 2009b). Therefore, when contrasted to the ~ 30 to 50% conception risk that is usually expected at the time of nonpregnancy diagnosis (NPD) 30 to 60 days after AI (Ferguson and Skidmore, 2013; Wiltbank and Pursley, 2014; Borchardt et al., 2017), it can be assumed that a substantial number of pregnancies are lost from the time of AI to NPD. Estimates of embryo loss from fertilization to Day 7, determined through the percentage of nonviable embryos recovered after uterine flushing, have ranged from 11 to 21 percentage points (Cerri et al., 2009a; Cerri et al., 2009b). Embryo losses before MRP have been attributed to deleterious genes, chromosome

aberrations, and poor oocyte quality (Hansen, 2002). Because these losses occur before, or right after the embryo enters the uterus, they are not expected to have any effect on the subsequent estrous cycle, and there is no known diagnostic method to detect these losses other than embryo recovery through uterine lavage.

4.2. Detection of embryo mortality and impact on the estrous cycle

It is commonly accepted that embryo demise around the period of MRP (14 to 19 days after AI) in cattle delays luteal regression (Humblot, 2001; Santos et al., 2004), thus, altering the normal pattern of return to estrus and ovulation which can be detrimental to estrus detection efficiency and the response of cows to resynchronization of ovulation protocols for timed AI (TAI). The greatest limitation to determine the effect of embryo loss on the estrous cycle has been lack of reliable methods to accurately estimate embryo mortality before 26 to 28 days after AI when the presence of an embryo can be detected by TUS (Pieterse et al., 1990; Filteau and Descôteaux, 1998). A caveat of the research that studied the effect of embryo mortality on ovarian function is that most studies estimated the occurrence of embryo mortality based on the inter-estrus intervals or P4 concentration dynamics. For example, Humblot (2001) suggested that cows undergoing embryo mortality before Day 16 would not have their estrous cycle extended, whereas cows undergoing embryo loss after Day 16 would have extended luteal phases (>24 days). The use of P4 concentration or delayed return to estrus as sole indicators of embryo mortality overestimates embryo losses because extended luteal phases can be a consequence of other phenomena such as variability among individuals or uterine infections. Also, return to estrus is not reliable because of limitations with estrus detection and the potential occurrence of ovulation without estrus expression (Valenza et al., 2012; Stevenson et al., 2014).

Alternative methods to provide an indication of the presence and subsequent demise of an embryo may be more accurate to determine embryo losses. In this regard, pregnancy associated glycoproteins (PAG) are a family of glycoproteins produced by the binucleate cells of the placenta (Butler et al., 1982; Xie et al., 1997; Klisch et al., 2006). These glycoproteins are produced and stored in granules within binucleate cells of the placenta, and released into the dam's blood stream after binucleate cells migrate and fuse with epithelial cells of the endometrium (Wooding, 1982). Currently, there is evidence of at least 22 members of this family of glycoproteins which are differentially expressed during gestation (Xie et al., 1991; Green et al., 2000; Patel et al., 2004). Although some of the available laboratory assays to detect PAG can identify specific members of the family (Zoli et al., 1992), other laboratory tests do not target a specific molecule, but rather recognize a group of them referred to as pregnancy specific protein B [PSPB; (Sasser et al., 1988)]. The fact that PAG are exclusively produced by the placenta makes them an accurate marker of pregnancy in cattle. In this regard, experiments conducted to validate the use of PAG as a method of pregnancy diagnosis showed high level of agreement between PAG concentrations and TUS to detect the presence of an embryo 27 to 35 days after AI (Szenci et al., 1998; Silva et al., 2007; Romano and Larson, 2010). Although PAG testing has been adopted as method for pregnancy testing in cattle, the methodology has some limitations. For example, the long half-life of PAG, which extends for as long as 70 days (Zoli et al., 1992; Kiracofe et al., 1993; Mialon et al., 1993) after calving and up to 10 d after embryo mortality during the first trimester of pregnancy (Szenci et al., 2000; Giordano et al., 2012b), can generate false pregnancy outcomes in the early postpartum period or in cows that undergo pregnancy loss.

The greatest limitation to the use PAG for determination of embryonic mortality around the early stages of the period of maternal recognition of pregnancy is the timing at which PAG

can be reliably detected in circulation with current laboratory assays. Although in some cows PAG can be detected as early as Day 15 of gestation (Sasser et al., 1986; Green et al., 2005), in most pregnant cows PAG cannot be detected until Day 25 (Whitlock and Maxwell, 2008). Several studies have reported PAG dynamics after spontaneous (Mialon et al., 1993; Szenci et al., 2000), and induced embryo mortality (Szenci et al., 2003; Giordano et al., 2012b), or used circulating PAG concentrations as a marker of late embryonic mortality (Humblot, 2001). Nevertheless, none of these experiments attempted to determine embryo loss around the early stages of the period of MRP. Mialon et al. (1993) measured serum concentrations of PAG in beef cows, beef heifers, and dairy cows at ~28, 35, 50 and 90 days after AI. Outcomes based on PAG were compared with NPD through TUS performed 50 days after AI and transrectal palpation performed 90 days after AI as reference test. In this experiment, they observed different PAG concentrations patterns among cows with late embryo loss. For example, a group of heifers had PAG concentrations indicative of the presence of an embryo on Day 28 but not on Day 35 (4/123). On the other hand, another group of heifers did not have elevated PAG concentrations on Day 28, but did on Day 35 (3/123). Moreover, among cows diagnosed nonpregnant 50 days after AI, a group of cows presented a slight increase (6/210) and another had declining PAG concentrations from Day 28 to Day 35 (15/210). Szenci et al., (2000) measured PAG and P4 concentrations on Day 21 after AI, every other day from 26 or 27 to 44 or 45, and on Day 53 or 58 in 11 cows that were diagnosed as undergoing embryo mortality using TUS. Data from this experiment showed great variation regarding time of luteal regression and disappearance of PAG in blood with regard to time of embryo death. Interestingly, in one group of cows the decline in PAG concentrations preceded embryo death, whereas in other the opposite was observed. Thus,

data from Szenci et al. (2000) suggested that PAG concentrations dynamics is not a reliable tool to diagnose timing of embryonic death.

Collectively, most data support the notion that circulating PAG concentration is a valuable marker of embryonic loss after PAG become detectable in circulation which, in most cows is not until after the final stages of the period of MRP. On the other hand, return to estrus and P4 concentration have been commonly used to estimate embryo mortality around the period of MRP. Nevertheless, these methods are not accurate because they are not pregnancy specific and are affected by additional physiological factors.

More recently, it has been demonstrated that a group of genes (interferon stimulated genes, ISG) are upregulated in peripheral white blood cells and other tissues in response to IFNT secreted by the embryo during the period of maternal recognition of pregnancy (Han et al., 2006; Gifford et al., 2007; Green et al., 2010). Gifford et al. (2007) performed an experiment designed to test the feasibility of identifying nonpregnant cows as early as Day 18 after AI based on mRNA expression of ISG in peripheral blood white cells. Lactating dairy cows (n = 50) received TAI and blood samples were collected at 0, 16, 18, and 20 days after AI. Transrectal ultrasonography on Day 40 to 45 was used as reference test. White blood cells were harvested and ISG mRNA expression was determined using RT-qPCR. Results from this experiment showed differential fold change in mRNA expression between pregnant and nonpregnant cows for certain ISG starting on Day 16 (myxovirus resistance protein 2, *MX2*) or 18 (interferon stimulated gene 15, *ISG15*) until Day 20 after AI.

Similar results were observed by Green et al. (2010) in three experiments performed to test different approaches for early NPD using ISG expression in dairy cows and heifers. In the first experiment, cows (n = 20) received TAI and white blood cells were obtained at 14, 16, 18,

and 20 days after AI to measure ISG expression. Nonpregnancy diagnosis was performed through TUS on Day 30 as reference test. Pregnant cows (n = 5) had greater *ISG15* and *MX2* mRNA expression (expressed as ratio to housekeeping gene) on Days 18 and 20 after AI. In the second experiment, mRNA was isolated from peripheral white blood cells on Day 17 (n = 32) or 18 (n = 42) after AI and NPD was performed on Day 32 by TUS. On Day 17, 2'-5'-oligoadenylate synthetase 1 (*Oas1*) mRNA expression (expressed as the ratio to housekeeping gene) was greater in pregnant than nonpregnant cows, whereas both *Oas1* and *MX2* were differentially expressed on Day 18. In addition, on Day 18 authors observed a pregnancy by parity interaction whereby pregnant multiparous cows had lower ISG expression levels than pregnant primiparous cows. In a third experiment, white blood cells were collected from lactating dairy cows (n = 54) and heifers (n = 24) during the luteal phase of the cycle preceding AI, and 18 days after AI to determine ISG mRNA expression as the ratio of the second sample to the first. For parous cows, there was no pregnancy or parity effect on *Oas1* or *MX2* mRNA expression, and there was a tendency for a pregnancy by parity interaction effect for the *MX2* ratio. On the other hand, ratios of *Oas1* and *MX2* were greater for pregnant than nonpregnant heifers. In these experiments, the authors also intended to establish a cut off value of ISG mRNA expression for NPD using ROC curves but they did not find a suitable value with acceptable sensitivity and specificity. Collectively, results from Gifford et al. (2007) and Green et al (2010) provide evidence that differential ISG mRNA expression in peripheral white blood cells between pregnant and nonpregnant cows may be used for early detection of the presence of an embryo.

Matsuyama et al. (2012) generated more evidence for ISG expression in response to IFNT in two experiments designed to evaluate the effect of different doses of intrauterine administration of IFNT on ISG mRNA expression in peripheral white blood cells. Moreover,

differences in ISG mRNA expression between non-lactating Japanese black cows that received AI, embryo transfer, or cyclic controls were explored. In the first experiment, at 16 or 17 days after estrus cows received intrauterine treatments of 0, 500 or 1,000 μg of recombinant IFNT. There was a treatment by time interaction on mRNA expression for *ISG15* and *MX2* in peripheral white blood cells whereby the greater doses of IFNT induced an increase in mRNA expression of greater magnitude and duration than the smallest dose. Interestingly, the correlation between dose of IFNT and mRNA expression was high (*ISG15*: r = 0.88, *MX2*: r = 0.83) suggesting that greater amount of IFNT released by embryos may be associated with greater mRNA expression of ISG in peripheral white blood cells. For the second experiment, blood samples were collected on Days 7, 16, 18, 21, and 25 after detected estrus (cyclic control cows; Day = 0), AI, or embryo transfer (ET; Day = 7). Pregnancy diagnosis through TUS 30 to 50 days after estrus was used as reference test. Cows were classified as pregnant (AI = 13 and ET = 16), nonpregnant with extended inter-estrus intervals (≥25 days; AI = 9 and ET = 11), nonpregnant with normal inter-estrus intervals (<25 days; AI = 19 and ET = 17), and cyclic controls (n = 15). Pregnant cows had greater *ISG15* mRNA expression than nonpregnant cows and cycling controls 18, 21, and 25 days after estrus. Interestingly, although non-statistically significant, ET cows with extended inter-estrus intervals had intermediate *ISG15* mRNA expression from 18 to 25 days after estrus suggesting that these cows had an embryo present around the time of MRP but the embryo did not survive until the time of pregnancy testing. Thus, it is possible to speculate that intermediate mRNA expression of *ISG15* might have been the result of lower IFNT production by a less viable embryo.

Collectively, results from these experiments provide evidence that differences in ISG mRNA expression in peripheral white blood cells between pregnant and nonpregnant cows may

be used for early detection of the presence of an embryo. Despite this, in these studies cows were retrospectively classified after NPD and none of the studies attempted to truly predict the presence of an embryo around the period of MRP.

Current data regarding embryo mortality around the period of maternal recognition of pregnancy and its impact on the ovarian dynamics is not consistent because most research used P4 concentration and delayed return to estrus as the diagnostic method. On the other hand, use of PAG and TUS as diagnostic methods provided evidence that embryo loss after Day 25 results in delayed luteal regression. The availability of new early pregnancy diagnostic methods such as ISG mRNA expression provide new tools to elucidate the real impact of embryo mortality before Day 25 on the estrous cycle.

5. Synchronization of ovulation in cattle

Synchronization of ovulation for TAI is one of the most widely applied biotechnologies in cattle production around the world (Lamb et al., 2010; Wiltbank and Pursley, 2014). Poor estrus detection efficiency due to physiological limitations of high producing dairy cows (Sangsritavong et al., 2002) or because of limited access to qualified labor and facilities make synchronization of ovulation a valuable tool to ensure timely insemination of cows. Moreover, some newly developed protocols may increase fertility to AI services as compared to insemination at detected estrus (Borchardt et al., 2016). The aim of synchronization of ovulation protocols is to manipulate follicular and luteal dynamics to facilitate the development of a dominant follicle in an ideal physiological environment and synchronize ovulation within a narrow time frame so that females can be inseminated at a predetermined time without the need for estrus detection.

The first synchronization of ovulation protocol reported in the literature (Pursley et al., 1995) consisted of a sequence of GnRH and PGF2α treatments (GnRH-7 days-PGF2α-2 days-GnRH). The first GnRH treatment given at random stages of the estrous cycle was aimed at inducing ovulation and the formation of a new CL. Also, the emergence of a new follicular wave is expected to happen 1.5 to 2 days after GnRH treatment (Pursley et al., 1995). Seven days after the first GnRH, cows are expected to have a dominant follicle and a mature CL (≥6 days old) that can undergo luteolysis in response to a single PGF2α treatment. After luteolysis, the dominant follicle is expected to continue growing so that 48 hours after the PGF2α treatment the GnRH-induced LH surge after the second GnRH treatment of the protocol induces ovulation within 24 to 32 hours in most cows (Pursley et al., 1995; Rantala et al., 2009; Giordano et al., 2012a). Thus, manipulating timing of ovulation allowed insemination of all cows at a fixed time without the need of performing estrus detection and attain similar fertility than that of cows inseminated upon estrus detection (Pursley et al., 1997). Following the development of the Ovsynch protocol, an overwhelming amount of research has been dedicated to develop and validate new synchronization protocols to maximize fertility through optimization of follicular development and the endocrine environment before and after insemination.

5.1. Synchronization of ovulation protocols for first AI postpartum

To maximize reproductive efficiency of dairy herds, cows need to be rapidly detected in estrus and inseminated after the end of the voluntary waiting period. In this regard, the adoption of synchronization of ovulation protocols ensures that cows are rapidly inseminated after the end of the voluntary waiting period without relying exclusively on estrus detection.

The main limitation of using the Ovsynch protocol alone to synchronize ovulation in dairy cows is that a considerable proportion of cows will not ovulate to the first GnRH treatment if the protocol is started at random stages of the estrous cycle (Pursley et al., 1995; Vasconcelos et al., 1999; Bello et al., 2006). This can happen because of: (1) absence of a follicle of sufficient size and maturity to respond to an LH surge, (2) presence of an already atretic dominant follicle, or (3) the occurrence of an LH surge of insufficient magnitude to cause ovulation. If cows fail to ovulate and a new follicular wave does not emerge ~1.5 to 2 days later, the dominant follicle present at the time of the first GnRH treatment may either ovulate spontaneously (in cows that do not have an active CL) or become atretic before the time of PGF2α treatment. In the first scenario, the newly formed CL will not be responsive to PGF2α at the time of treatment, and in both cases there will probably not be a dominant follicle capable of ovulating at the time of second GnRH treatment. Moreover, in some cows the follicular wave giving rise to the ovulatory follicle may emerge shortly before the first GnRH treatment. In consequence, these cows would ovulate an "old" follicle. This phenomenon has been associated with lower fertility probably due to reduced oocyte quality (Revah and Butler, 1996) which leads to reduced embryo quality (Cerri et al., 2009b). Further, cows that ovulate to the first GnRH treatment of Ovsynch-like protocols have greater fertility than cows that do not (Chebel et al., 2003; Galvão et al., 2007; Bisinotto et al., 2010), probably because of better synchronization of the follicular wave. In this regard, there is evidence that initiating the Ovsynch protocol on Days 5 to 9 of the estrous cycle maximizes the number of cows that have their follicular wave synchronized (Vasconcelos et al., 1999; Bello et al., 2006). Therefore, with the objective of maximizing the proportion of cows that are at an optimal stage of the estrous cycle at the time of the first GnRH treatment, presynchronization treatments have been developed.

The Presynch-Ovsynch protocol was the first presynchronization treatment described in the literature (Moreira et al., 2001). It consisted of two PGF2α treatments 14 days apart, and the first GnRH treatment of Ovsynch given 12 days after the second PGF2α. The reasoning behind this treatment is that cows bearing a mature CL at the time of first PGF2α treatment would show estrus within a seven day period, hence at the time of the second PGF2α treatment they would be expected to have a seven day old or older CL which could respond to the second PGF2α treatment. Because cows that fail to respond to the first treatment would be probably in metestrus or proestrus, they would be expected to be in diestrus at the time of the second PGF2α treatment. Thus, most cows would be expected to have a mature CL at the time of second PGF2α treatment, and thereby would be expected to undergo luteolysis, display estrus, and ovulate within three to seven days after treatment. Because of synchronized estrus and ovulation after the Presynch portion of the protocol, most cows would be on Days 5 to 9 of the estrous cycle at the time of first GnRH of Ovsynch which increases the response to the GnRH treatment and the rest of the protocol. The improvement in synchrony of ovulation for cows that received the presynchronization protocol resulted in greater pregnancy per AI (P/AI; 48.5%) than for cows that only received the Ovsynch protocol [36.6%, (Moreira et al., 2001)].

Bello et al. (2006) tested a protocol that included PGF2α and GnRH to presynchronize cows for first service postpartum. The Ovsynch protocol without presynchronization was compared with a presynchronization treatment that consisted of a PGF2α treatment followed by GnRH two days later. The Ovsynch protocol was initiated either four (G4G), five (G5G) or six (G6G) days after the GnRH for presynchronization. Initiating Ovsynch 6 d after the presynchronization treatment maximized the proportion of cows that ovulated in response to the first GnRH of Ovsynch (Ovsynch = 54%, G4G = 56%, G5G = 67%, and G6G = 85%), optimized

the response to the PGF2α of Ovsynch (Ovsynch = 69%, G4G = 92%, G5G = 92%, and G6G = 96%), and resulted in the greatest synchronization rate (Ovsynch = 69%, G4G = 80%, G5G = 75%, and G6G = 92%). This improved response to the G6G protocol translated into greater P/AI than for presynchronized and not presynchronized cows (Ovsynch = 27%, G4G = 24%, G5G = 39%, and G6G = 50%), thereby it was concluded that G6G could be used to improve the fertility of first AI service in lactating dairy cows.

More recently, Souza et al. (2008) developed the Double-Ovsynch protocol which consists of two Ovsynch protocols seven days apart. The first Ovsynch, usually referred to as Pre-Ovsynch, not only sets most cows to Day 7 of the estrous cycle at the time of first GnRH of Ovsynch but also induces ovulation in most anovular cows (Souza et al., 2008; Herlihy et al., 2012; Ayres et al., 2013). This is an advantage compared with PGF2α-based protocols such as Presynch-Ovsynch because in the latter, anovular cows usually do not respond to the presynchronization protocol. In fact, the proportion of cows with P4 concentration indicative of a functional CL at the time of first GnRH of Ovsynch was greater for cows that received the Double-Ovsynch (90.6%) than the Presynch-Ovsynch protocol [66.6% (Souza et al., 2008)]. Further, Double-Ovsynch also increased the proportion of cows with high P4 (≥ 3 ng/mL) at the time of PGF2α treatment of Ovsynch. Increased synchrony of ovulation and improved endocrine environment before TAI translated into 8 percentage points greater P/AI in cows treated with Double-Ovsynch than the Presynch-Ovsynch protocol (Souza et al., 2008).

These synchronization protocols designed to increase P/AI to first service have been widely adopted by the dairy industry in the U.S. (Caraviello et al., 2006; Ferguson and Skidmore, 2013; Scott, 2016). In combination with improvements in cow genetics and herd management

adoption of fertility programs allows high producing dairy herds to achieve first service P/AI of up to ~50% (Giordano et al., 2012e; Herlihy et al., 2012; Borchardt et al., 2017).

5.2. Management strategies for second and greater AI services.

Even though the combination of synchronization programs for first service and overall improvements in herd management allow well-managed farms to achieve P/AI in the range of 40 to 55% (Souza et al., 2008; Borchardt et al., 2016; Borchardt et al., 2017), there is a substantial proportion of nonpregnant cows that needs rapid re-insemination. In this regard, a common reproductive management strategy for second and greater AI services consists of the combination of insemination after estrus detection and a resynchronization of ovulation protocol for TAI for cows not re-inseminated at detected estrus. The most commonly used protocol for resynchronization of ovulation and TAI is Ovsynch (Caraviello et al., 2006; Scott, 2016) which, when used for resynchronization, is commonly referred to as Resynch.

The main objective of resynchronizing cows is to minimize time to pregnancy after the end of the voluntary waiting period, hence maximizing reproductive efficiency and herd profitability (Giordano et al., 2013). Time to pregnancy is determined by the insemination and conception risk which can be increased by reducing the interbreeding interval and maximizing P/AI, respectively. A potential strategy for minimizing the interbreeding interval is to initiate Resynch protocols as early as possible after a previous AI. The possibility of treating pregnant cows with GnRH allows to initiate resynchronization of ovulation protocols before NPD, and the use of TUS allows to perform NPD as early as 26 days after AI (Pieterse et al., 1990; Filteau and Descôteaux, 1998). Therefore, Resynch protocols can be initiated as early as 19 days after AI (Fricke et al., 2003). Despite this, other factors such as: (1) the expected physiological response

to treatments, (2) impact on estrus detection, and (3) the feasibility of performing an accurate NPD must be taken into account when deciding the timing of initiation of a resynchronization protocol.

Fricke et al. (2003) evaluated the impact of initiating resynchronization of ovulation at three different intervals after AI on conception risk in lactating dairy cows. Cows were initiated in the Cosynch protocol (GnRH-7 d-PGF2α-2 d-GnRH + TAI) 19 (D19), 26 (D26), or 33 (D33) days after AI. Pregnancy per AI was greater for cows in the D26 and D33 treatments (34 and 38% respectively) than for the D19 treatment (23%). Therefore, although the D19 treatment minimized the interbreeding interval and increased insemination risk, it did not maximize conception risk. It was suggested that the greater proportion of cows expected to be in proestrus at the time of the first GnRH treatment in the D19 treatment, may have resulted in an inferior ovulatory response and synchronization rate, which in turn, resulted in lower fertility. Furthermore, authors observed greater pregnancy loss when NPD was performed 26 days after AI (D19 and D26 treatments) than 33 days after AI (D33 treatment). Thus, although the D19 and D26 treatments maximized insemination risk, the reduction in fertility and increased pregnancy losses may offset the beneficial impact of shorter interbreeding interval on pregnancy rate. Lopes et al. (2013) performed another experiment to compare P/AI after initiating resynch 32 ± 3 (R32) and 39 ± 3 (R39) days after AI. No difference in P/AI was observed among treatments (R32 = 33.6% and R39 = 34.3%). In summary, resynchronization of ovulation protocols initiated at 26, 32, and 39 days after AI result in similar conception risks.

An important limitation to the experiments described above is that the impact of initiating resynch before NPD on estrus expression was not evaluated. Maximizing the proportion of cows re-inseminated upon estrus detection reduces the interbreeding interval and treatment costs. In

this regard, the proportion of cows re-inseminated at detected estrus before NPD was evaluated after initiating Resynch 21 and 28 days after AI (Chebel et al., 2003) or 24 and 31 days after AI (Bruno et al., 2014). In both experiments, there was no effect of the day of initiation of Resynch on P/AI, however, the proportion of cows re-inseminated upon estrus detection was reduced by the early GnRH treatment. Limited estrus expression after GnRH treatment can be attributed to the suppression of E2 production by granulosa cells of the dominant follicle in response to the GnRH-induced LH surge (Berndtson et al., 1995; Komar et al., 2001; Jo and Fortune, 2003). Thus, treatment of cows with GnRH around the time when most cows are expected to express estrous behavior after a previous insemination increases the number of cows submitted to TAI, increases reproductive treatment costs, and extends the interbreeding interval.

Presynchronization protocols for first service TAI increase conception risk by maximizing the proportion of cows that are at an ideal stage of the estrous cycle at the time of the first GnRH of Ovsynch. Likewise, extensive research has been done to improve conception risk after resynchronization of ovulation protocols using different presynchronization strategies. Giordano et al. (2012d) performed two experiments to evaluate different approaches for presynchronization before Resynch. In the first experiment, they compared the Day-25 Resynch protocol (D25; Ovsynch initiated 25 days after AI) without presynchronization, the D25 protocol with a human chorionic gonadotropin (hCG) treatment on Day 18 after AI (HGPG), and the Double-Ovsynch (DO) protocol initiated 22 days after AI. Nonpregnancy diagnosis was performed 32 days after AI. Pregnancy per AI 53 days after AI was greater for the HGPG (33.4%) and DO (32.5%) groups than for the D25 group (25.4%). This first experiment provided evidence that improving synchrony of ovulation and the endocrine environment (mainly P4 concentration) at the time of the first GnRH of Resynch has a positive impact on fertility.

Taken together, most of the available evidence shows that the time of initiation of resynchronization of ovulation protocols does not have a substantial impact on conception risk after TAI and that using a presynchronization strategy may increase conception risk after TAI. Despite this, these experiments were focused on the effect of treatments on P/AI without assessing the impact on time to pregnancy, which is the final driver of reproductive efficiency. Moreover, protocols initiated prior to NPD negatively affected estrus expression, which in turn can negatively affect insemination risk. Further, part of the improvement on P/AI obtained with presynchronization protocols for resynchronization of ovulation may be due to forcing cows with high fertility to be inseminated at TAI because of estrus suppression. Considering that many dairy farms are adopting new technologies to assist with detection of estrus or may have an interest in increasing the proportion of cows submitted for AI after a detected estrus, it is paramount to develop resynchronization of ovulation protocols that do not interfere with estrus expression after a previous insemination while minimizing the interbreeding interval and the number of unnecessary treatments.

5.3. Role of progesterone at the time of initiating synchronization of ovulation protocols and during follicular development.

Cows not bearing a CL or with low circulating P4 concentration (< 1 ng/mL) at the time of the first GnRH of Ovsynch have reduced conception risk compared with cows with a CL and high P4 concentration (Sterry et al., 2006; Herlihy et al., 2012; Bisinotto et al., 2015a). Ovulatory response to the GnRH treatment, which is needed for initiation of a new follicular wave, is greater when P4 concentration is low at the time of treatment (Giordano et al., 2012a; Stevenson and Pulley, 2016). Despite this, fertility seems to be more dependent on high P4 concentration at

the time of first GnRH treatment and during follicular development than on ovulatory response (Stevenson and Lamb, 2016). This is probably because follicles that develop in a low P4 environment might have compromised oocyte quality (Rivera et al., 2011) due to prolonged exposure to high frequency and low magnitude pulses of LH (Endo et al., 2012) which, in turn, can cause asynchrony between oocyte maturation and ovulation (Revah and Butler, 1996; Inskeep, 2004). It is also possible that low circulating P4 concentration between the first GnRH and the PGF2α treatment of Ovsynch-like protocols is associated with a short estrous cycle after insemination due to earlier upregulation of estradiol receptors in the endometrium (Cerri et al., 2011). Earlier luteolysis might then impair fertility by triggering the luteolytic cascade before establishment of MRP.

The impact of P4 supplementation during Ovsynch-like protocols has been studied extensively. A recent meta-analysis from Bisinotto et al. (2015b) reported that P4 supplementation using controlled intravaginal release devices in cows without a CL at the time of the first GnRH of Ovsynch resulted in a 5.8 percentage point increment in P/AI [31.1% (n = 2,071) vs 36.9 (n = 2,117) for untreated and treated cows, respectively]. Conversely, no major benefit was observed for cows bearing a CL at the initiation of the protocol [39.4% (n = 4,808) vs 40.9 (n = 4,766) for untreated and treated cows].

5.4. Progesterone concentration at the time of GnRH treatment before timed AI and at the time of timed AI.

The aim of the GnRH treatment before TAI is to induce an LH surge which, in turn, will trigger ovulation within 28 to 32 hours after treatment (Pursley et al., 1995). Lack of complete luteal regression by the time of the GnRH treatment before TAI results in elevated circulating P4

concentration and an LH surge of lower magnitude (Giordano et al., 2012a; Stevenson and Pulley, 2016) which reduces the risk of ovulation around TAI (Stevenson and Pulley, 2016). Therefore, minimizing circulating P4 concentration at the time of GnRH treatment before TAI is paramount to maximize the number of cows that ovulate within the expected time frame and thereby maximize fertility to TAI. Moreover, data from rats suggest that P4 can be detrimental for oocyte transport in the oviduct (Fuentealba et al., 1988) which may explain part of the detrimental effect of high P4 at the time of AI.

Data from three field studies support the hypothesis that low P4 concentration (< 0.5 ng/mL) at the time of AI result in greater conception risk in dairy heifers (De Silva et al., 1981) and lactating dairy cows (Waldmann et al., 2001; Ghanem et al., 2006) inseminated at detected estrus. Further, similar results were observed in cows synchronized with different Ovsynch-like protocols in which P4 concentration at the time of GnRH or TAI below 0.5 ng/mL was associated with greater conception risk (Giordano et al., 2012e; Lopes et al., 2013; Stevenson and Lamb, 2016).

Progesterone concentration at TAI in synchronization of ovulation protocols depends on the luteolytic response to the PGF2α treatment. Luteal regression after a single PGF2α treatment in synchronization of ovulation protocols ranges from 70 to 94% (Moreira et al., 2000; Brusveen et al., 2009; Martins et al., 2011). Therefore, 6 to 30% of the cows have P4 concentration above the optimal level at the time of TAI and will have compromised fertility. The presence of a young CL (≤6 days old) not fully responsive to PGF2α has been proposed as the main reason for lack of CL regression (Henricks et al., 1974; Wiltbank et al., 2015). Failure to induce luteal regression appears to be more frequent in resynchronization of ovulation protocols than in cows synchronized for first AI postpartum (Martins et al., 2011). Further, Giordano et al. (2012e)

observed that luteal regression was compromised among cows than did not have a CL at the time of initiating Resynch and ovulated in response to GnRH than cows with a functional CL. Only 35.6% of cows that ovulated after the first GnRH treatment had P4 <0.4 ng/mL 56 h after induction of ovulation with PGF2α before TAI.

Given the importance of minimizing circulating P4 around induction of ovulation for TAI, different alternatives have been evaluated to ensure complete luteal regression. For example, a second PGF2α treatment 24 h after induction of luteal regression in Ovsynch-like protocols has been shown to increase the proportion of cows with complete luteal regression at the time of GnRH treatment and TAI (Brusveen et al., 2009; Carvalho et al., 2015; Wiltbank et al., 2015). Moreover, Carvalho et al. (2015) observed greater P/AI when using two PGF2α treatments 24 hours apart than when using a single treatment (39 vs. 33%). This increase in P/AI was attributed to a greater proportion of cows with complete luteal regression in the group that received two PGF2α treatments (98%) than in the group that received a single PGF2α (83%). Moreover, Santos et al. (2010) reported greater risk of luteolysis after two PGF2α treatments 24 hours apart (95.7%) than a single treatment (59.1%) in a five day-Cosynch protocol (GnRH-5 d-PGF2α-72 h-GnRH + TAI). These results provide evidence that a second PGF2α treatment is effective to induce complete luteal regression even for cows with young CLs not fully responsive to a single PGF2α treatment.

6. Summary

This literature review is intended to give an overview of the main physiological mechanisms that govern the estrous cycle in the cow and current knowledge of the effect of embryonic mortality on ovarian function. Moreover, it summarizes current research regarding

synchronization of ovulation protocols and important physiological aspects that determine the effectiveness of these protocols.

Diagnostic methods used to detect embryo mortality in experiments aimed at elucidating the effect of this phenomenon on ovarian dynamics did not have the necessary accuracy to detect the presence of an embryo prior to Day 24 after AI. Therefore, more research is needed to understand the effect of early embryo mortality occurring around the period of MRP on ovarian dynamics. In this regard, determination of ISG mRNA expression in peripheral white blood cells is a promising tool to detect the presence of an embryo during the period of MRP. Thus, the experiment presented in Chapter II of this section had the objective of identifying embryo mortality around the period of MRP and determine its impact on ovarian function in lactating dairy cows. Thereafter, Chapters III and IV present data from experiments conducted to evaluate new programs for resynchronization of ovulation. These programs were aimed at minimizing time to pregnancy during lactation reducing the interbreeding interval for TAI services and increasing P/AI for cows expected to have low fertility after TAI while not interfering with estrus expression after a previous insemination. These programs were designed based on our improved understanding of the ovarian dynamics of lactating dairy cows after a previous insemination (Chapter II of this section).

7. References

Adams, G., R. Jaiswal, J. Singh, and P. Malhi. 2008. Progress in understanding ovarian follicular dynamics in cattle. Theriogenology 69:72-80.

Aerts, J. and P. Bols. 2010. Ovarian follicular dynamics. A review with emphasis on the bovine species. Part II: Antral development, exogenous influence and future prospects. Reproduction in domestic animals 45:180-187.

Araujo, R. R., O. Ginther, J. C. Ferreira, M. M. Palhão, M. A. Beg, and M. C. Wiltbank. 2009. Role of follicular estradiol-17beta in timing of luteolysis in heifers. Biology of reproduction 81:426-437.

Ayalon, N. 1978. A review of embryonic mortality in cattle. Journal of Reproduction and Fertility 54:483-493.

Ayres, H., R. Ferreira, A. Cunha, R. Araújo, and M. Wiltbank. 2013. Double-Ovsynch in high-producing dairy cows: Effects on progesterone concentrations and ovulation to GnRH treatments. Theriogenology 79:159-164.

Bazer, F. W., T. E. Spencer, and T. L. Ott. 1997. Interferon tau: a novel pregnancy recognition signal. American Journal of Reproductive Immunology 37:412-420.

Bello, N. M., J. Steibel, and J. Pursley. 2006. Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of Ovsynch in lactating dairy cows. Journal of dairy science 89:3413-3424.

Berndtson, A., S. Vincent, and J. Fortune. 1995. Low and high concentrations of gonadotropins differentially regulate hormone production by theca interna and granulosa cells from bovine preovulatory follicles. Biology of reproduction 52:1334-1342.

Bisinotto, R., L. Castro, M. Pansani, C. Narciso, N. Martinez, L. Sinedino, T. Pinto, N. Van de Burgwal, H. Bosman, and R. Surjus. 2015a. Progesterone supplementation to lactating dairy cows without a corpus luteum at initiation of the Ovsynch protocol. Journal of dairy science 98:2515-2528.

Bisinotto, R., R. Chebel, and J. Santos. 2010. Follicular wave of the ovulatory follicle and not cyclic status influences fertility of dairy cows. Journal of dairy science 93:3578-3587.

Bisinotto, R., I. Lean, W. Thatcher, and J. Santos. 2015b. Meta-analysis of progesterone supplementation during timed artificial insemination programs in dairy cows. Journal of dairy science 98:2472-2487.

Boos, A., W. Meyer, R. Schwarz, and E. Grunert. 1996. Immunohistochemical assessment of oestrogen receptor and progesterone receptor distribution in biopsy samples of the bovine endometrium collected throughout the oestrous cycle. Animal Reproduction Science 44:11-21.

Borchardt, S., P. Haimerl, and W. Heuwieser. 2016. Effect of insemination after estrous detection on pregnancy per artificial insemination and pregnancy loss in a Presynch-Ovsynch protocol: A meta-analysis. Journal of dairy science 99:2248-2256.

Borchardt, S., P. Haimerl, A. Pohl, and W. Heuwieser. 2017. Evaluation of prostaglandin F 2α versus prostaglandin F 2α plus gonadotropin-releasing hormone as Presynch methods preceding an Ovsynch in lactating dairy cows: A meta-analysis. Journal of Dairy Science 100:4065-4077.

Brännström, M. u. and R. Norman. 1993. Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function. Human Reproduction 8:1762-1775.

Bruno, R., J. Moraes, J. Hernández-Rivera, K. Lager, P. Silva, A. Scanavez, L. Mendonça, R. Chebel, and T. Bilby. 2014. Effect of an Ovsynch56 protocol initiated at different intervals after insemination with or without a presynchronizing injection of gonadotropin-releasing hormone on fertility in lactating dairy cows. Journal of dairy science 97:185-194.

Brusveen, D., A. Souza, and M. Wiltbank. 2009. Effects of additional prostaglandin F 2α and estradiol-17β during Ovsynch in lactating dairy cows. Journal of dairy science 92:1412-1422.

Butler, J., W. Hamilton, R. Sasser, C. Ruder, G. Hass, and R. Williams. 1982. Detection and partial characterization of two bovine pregnancy-specific proteins. Biology of Reproduction 26:925-933.

Caraviello, D., K. Weigel, P. Fricke, M. Wiltbank, M. Florent, N. Cook, K. Nordlund, N. Zwald, and C. Rawson. 2006. Survey of management practices on reproductive performance of dairy cattle on large US commercial farms. Journal of dairy science 89:4723-4735.

Carvalho, P., M. Fuenzalida, A. Ricci, A. Souza, R. Barletta, M. Wiltbank, and P. Fricke. 2015. Modifications to Ovsynch improve fertility during resynchronization: Evaluation of presynchronization with gonadotropin-releasing hormone 6 d before initiation of Ovsynch and addition of a second prostaglandin F2α treatment. Journal of dairy science 98:8741-8752.

Cerri, R., R. Chebel, F. Rivera, C. Narciso, R. Oliveira, M. Amstalden, G. Baez-Sandoval, L. Oliveira, W. Thatcher, and J. Santos. 2011. Concentration of progesterone during the development of the ovulatory follicle: II. Ovarian and uterine responses. Journal of dairy science 94:3352-3365.

Cerri, R., S. Juchem, R. Chebel, H. Rutigliano, R. Bruno, K. Galvao, W. Thatcher, and J. Santos. 2009a. Effect of fat source differing in fatty acid profile on metabolic parameters, fertilization, and embryo quality in high-producing dairy cows. Journal of Dairy Science 92:1520-1531.

Cerri, R. L., H. M. Rutigliano, R. C. Chebel, and J. E. Santos. 2009b. Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. Reproduction 137:813-823.

Chebel, R. C., J. E. Santos, R. L. Cerri, K. N. Galvão, S. O. Juchem, and W. W. Thatcher. 2003. Effect of resynchronization with GnRH on day 21 after artificial insemination on pregnancy rate and pregnancy loss in lactating dairy cows. Theriogenology 60:1389-1399.

Crowe, M. 1999. Gonadotrophs Control of Terminal Follicular Growth in Cattle. Reproduction in Domestic Animals 34:157-166.

Davis, A., I. Fleet, F. Harrison, and F. M. Walker. 1980. Pulmonary metabolism of prostaglandin F2a in the conscious non-pregnant ewe and sow. Journal of physiology.

De Vries, A. 2006. Economic value of pregnancy in dairy cattle. Journal of dairy science 89:3876-3885.

Diskin, M. and D. Morris. 2008. Embryonic and early foetal losses in cattle and other ruminants. Reproduction in Domestic Animals 43:260-267.

Endo, N., K. Nagai, T. Tanaka, and H. Kamomae. 2012. Profiles of ovarian steroids, luteinizing hormone and estrous signs from luteolysis to ovulation in lactating and non-lactating dairy cows. Journal of Reproduction and Development 58:685-690.

Farin, C., K. Imakawa, T. Hansen, J. McDonnell, C. Murphy, P. Farin, and R. Roberts. 1990. Expression of trophoblastic interferon genes in sheep and cattle. Biology of Reproduction 43:210-218.

Ferguson, J. D. and A. Skidmore. 2013. Reproductive performance in a select sample of dairy herds. Journal of dairy science 96:1269-1289.

Filteau, V. and L. DesCôteaux. 1998. Predictive values of early pregnancy diagnosis by ultrasonography in dairy cattle. in Proc. American Association of Bovine Practitioners. Conference (USA).

Flint, A., E. Sheldrick, T. McCann, and D. Jones. 1990. Luteal oxytocin: characteristics and control of synchronous episodes of oxytocin and PGF2α secretion at luteolysis in ruminants. Domestic animal endocrinology 7:111-124.

Forde, N., P. A. McGettigan, J. P. Mehta, L. O'Hara, S. Mamo, F. W. Bazer, T. E. Spencer, and P. Lonergan. 2014. Proteomic analysis of uterine fluid during the pre-implantation period of pregnancy in cattle. Reproduction 147:575-587.

Fortune, J. 1994. Ovarian follicular growth and development in mammals. Biology of reproduction 50:225-232.

Fortune, J., G. Rivera, and M. Yang. 2004. Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. Animal Reproduction Science 82:109-126.

Fricke, P., D. Caraviello, K. Weigel, and M. Welle. 2003. Fertility of dairy cows after resynchronization of ovulation at three intervals following first timed insemination. Journal of dairy science 86:3941-3950.

Fuentealba, B., M. Nieto, and H. Croxatto. 1988. Progesterone abbreviates the nuclear retention of estrogen receptor in the rat oviduct and counteracts estrogen action on egg transport. Biology of reproduction 38:63-69.

Galvão, K., M. Sá Filho, and J. Santos. 2007. Reducing the interval from presynchronization to initiation of timed artificial insemination improves fertility in dairy cows. Journal of dairy science 90:4212-4218.

Geisert, R., M. Zavy, B. Biggers, J. Garrett, and R. Wettemann. 1988. Characterization of the uterine environment during early conceptus expansion in the bovine. Animal Reproduction Science 16:11-25.

Ghanem, M., T. Nakao, K. Nakatani, M. Akita, and T. Suzuki. 2006. Milk Progesterone Profile at and after Artificial Insemination in Repeat-Breeding Cows: Effects on Conception Rate and Embryonic Death. Reproduction in Domestic Animals 41:180-183.

Gifford, C. A., K. Racicot, D. S. Clark, K. J. Austin, T. R. Hansen, M. C. Lucy, C. J. Davies, and T. L. Ott. 2007. Regulation of interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant dairy cows. J Dairy Sci 90:274-280.

Ginther, O., R. Araujo, M. Palhao, B. Rodrigues, and M. Beg. 2009. Necessity of Sequential Pulses of Prostaglandin F2alpha for Complete Physiologic Luteolysis in Cattle 1. Biology of reproduction 80:641-648.

Ginther, O., M. Wiltbank, P. Fricke, J. Gibbons, and K. Kot. 1996. Selection of the dominant follicle in cattle. Biology of reproduction 55:1187-1194.

Giordano, J., P. Fricke, and V. Cabrera. 2013. Economics of resynchronization strategies including chemical tests to identify nonpregnant cows. Journal of dairy science 96:949-961.

Giordano, J., P. Fricke, J. Guenther, G. Lopes, M. Herlihy, A. Nascimento, and M. Wiltbank. 2012a. Effect of progesterone on magnitude of the luteinizing hormone surge induced by two different doses of gonadotropin-releasing hormone in lactating dairy cows. Journal of dairy science 95:3781-3793.

Giordano, J., P. Fricke, M. Wiltbank, and V. Cabrera. 2011. An economic decision-making support system for selection of reproductive management programs on dairy farms. Journal of dairy science 94:6216-6232.

Giordano, J., J. Guenther, G. Lopes, and P. Fricke. 2012b. Changes in serum pregnancy-associated glycoprotein, pregnancy-specific protein B, and progesterone concentrations before and after induction of pregnancy loss in lactating dairy cows. Journal of dairy science 95:683-697.

Giordano, J., A. Kalantari, P. Fricke, M. Wiltbank, and V. Cabrera. 2012c. A daily herd Markov-chain model to study the reproductive and economic impact of reproductive programs combining timed artificial insemination and estrus detection. Journal of dairy science 95:5442-5460.

Giordano, J., M. Wiltbank, J. Guenther, M. Ares, G. Lopes, M. Herlihy, and P. Fricke. 2012d. Effect of presynchronization with human chorionic gonadotropin or gonadotropin-releasing hormone 7 days before resynchronization of ovulation on fertility in lactating dairy cows. Journal of dairy science 95:5612-5625.

Giordano, J., M. Wiltbank, J. Guenther, R. Pawlisch, S. Bas, A. Cunha, and P. Fricke. 2012e. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. Journal of dairy science 95:639-653.

Godkin, J., F. Bazer, W. Thatcher, and R. Roberts. 1984. Proteins released by cultured day 15–16 conceptuses prolong luteal maintenance when introduced into the uterine lumen of cyclic ewes. Journal of reproduction and fertility 71:57-64.

Godkin, J. D., B. Lifsey, and B. Gillespie. 1988. Characterization of bovine conceptus proteins produced during the peri-and postattachment periods of early pregnancy. Biology of reproduction 38:703-711.

Goodman, R. and E. Inskeep. 2006. Neuroendocrine control of the ovarian cycle of the sheep. Knobil and Neill's physiology of reproduction 3:2389-2447.

Green, J., C. Okamura, S. Poock, and M. Lucy. 2010. Measurement of interferon-tau (IFN-τ) stimulated gene expression in blood leukocytes for pregnancy diagnosis within 18–20d after insemination in dairy cattle. Animal reproduction science 121:24-33.

Green, J. A., T. E. Parks, M. P. Avalle, B. P. Telugu, A. L. McLain, A. J. Peterson, W. McMillan, N. Mathialagan, R. R. Hook, and S. Xie. 2005. The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. Theriogenology 63:1481-1503.

Green, J. A., S. Xie, X. Quan, B. Bao, X. Gan, N. Mathialagan, J.-F. Beckers, and R. M. Roberts. 2000. Pregnancy-Associated Bovine and Ovine Glycoproteins Exhibit Spatially and Temporally Distinct Expression Patterns During Pregnancy 1. Biology of Reproduction 62:1624-1631.

Han, H., K. J. Austin, L. A. Rempel, and T. R. Hansen. 2006. Low blood ISG15 mRNA and progesterone levels are predictive of non-pregnant dairy cows. J Endocrinol 191:505-512.

Hansen, P. 2002. Embryonic mortality in cattle from the embryo's perspective. Journal of Animal Science 80:E33-E44.

Henricks, D., J. Long, J. Hill, and J. Dickey. 1974. The effect of prostaglandin F2α during various stages of the oestrous cycle of beef heifers. Journal of reproduction and fertility 41:113-120.

Herlihy, M., J. Giordano, A. Souza, H. Ayres, R. Ferreira, A. Keskin, A. Nascimento, J. Guenther, J. Gaska, and S. Kacuba. 2012. Presynchronization with Double-Ovsynch improves fertility at first postpartum artificial insemination in lactating dairy cows. Journal of dairy science 95:7003-7014.

Hubbert, W. 1972. Committee on bovine reproductive nomenclature. Recommendations for standardizing bovine reproductive terms. Cornell Vet 62:216-237.

Hue, I., S. Degrelle, E. Campion, and J. Renard. 2007. Gene expression in elongating and gastrulating embryos from ruminants. Society of Reproduction and Fertility supplement 64:365.

Humblot, P. 2001. Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. Theriogenology 56:1417-1433.

Inskeep, E. 2004. Preovulatory, postovulatory, and postmaternal recognition effects of concentrations of progesterone on embryonic survival in the cow. Journal of animal science 82:E24-E39.

Ireland, J., M. Mihm, E. Austin, M. Diskin, and J. Roche. 2000. Historical perspective of turnover of dominant follicles during the bovine estrous cycle: key concepts, studies, advancements, and terms. Journal of Dairy Science 83:1648-1658.

Jeong, K. and U. Kaiser. 2006. Gonadotropin-releasing hormone regulation of gonadotropin biosynthesis and secretion. Knobil and Neill's physiology of reproduction. USA: Elsevier:1635-1701.

Jo, M. and J. Fortune. 2003. Changes in oxytocin receptor in bovine preovulatory follicles between the gonadotropin surge and ovulation. Molecular and cellular endocrinology 200:31-43.

Kimmins, S. and L. MacLaren. 2001. Oestrous cycle and pregnancy effects on the distribution of oestrogen and progesterone receptors in bovine endometrium. Placenta 22:742-748.

Kiracofe, G., J. Wright, R. Schalles, C. Ruder, S. Parish, and R. Sasser. 1993. Pregnancy-specific protein B in serum of postpartum beef cows. Journal of animal science 71:2199-2205.

Klisch, K., A. Boos, M. Friedrich, K. Herzog, M. Feldmann, N. Sousa, J.-F. Beckers, R. Leiser, and G. Schuler. 2006. The glycosylation of pregnancy-associated glycoproteins and prolactin-related protein-I in bovine binucleate trophoblast giant cells changes before parturition. Reproduction 132:791-798.

Knickerbocker, J., W. Thatcher, F. Bazer, M. Drost, D. Barron, K. Fincher, and R. Roberts. 1986. Proteins secreted by day-16 to-18 bovine conceptuses extend corpus luteum function in cows. Journal of reproduction and fertility 77:381-391.

Komar, C., A. Berndtson, A. Evans, and J. Fortune. 2001. Decline in Circulating Estradiol During the Periovulatory Period Is Correlated with Decreases in Estradiol and Androgen, and in Messenger RNA for P450 Aromatase and P450 17α-Hydroxylase, in Bovine Preovulatory Follicles 1. Biology of reproduction 64:1797-1805.

Lamb, G., C. Dahlen, J. Larson, G. Marquezini, and J. Stevenson. 2010. Control of the estrous cycle to improve fertility for fixed-time artificial insemination in beef cattle: a review. Journal of animal science 88:E181-E192.

Lamming, G., D. Wathes, A. Flint, J. Payne, K. Stevenson, and J. Vallet. 1995. Local action of trophoblast interferons in suppression of the development of oxytocin and oestradiol receptors in ovine endometrium. Journal of reproduction and fertility 105:165-175.

Lee, J., J. A. McCracken, S. K. Banu, R. Rodriguez, T. K. Nithy, and J. A. Arosh. 2010. Transport of prostaglandin F2α pulses from the uterus to the ovary at the time of luteolysis in ruminants is regulated by prostaglandin transporter-mediated mechanisms. Endocrinology 151:3326-3335.

Leung, S. and D. Wathes. 2000. Oestradiol regulation of oxytocin receptor expression in cyclic bovine endometrium. Journal of reproduction and fertility 119:287-292.

Lopes, G., J. Giordano, A. Valenza, M. Herlihy, J. Guenther, M. Wiltbank, and P. Fricke. 2013. Effect of timing of initiation of resynchronization and presynchronization with gonadotropin-releasing hormone on fertility of resynchronized inseminations in lactating dairy cows. Journal of dairy science 96:3788-3798.

Mann, G. and G. Lamming. 1994. Use of repeated biopsies to monitor endometrial oxytocin receptors in the cow. The Veterinary record 135:403-405.

Mann, G. and G. Lamming. 2001. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction 121:175-180.

Martins, J., R. Policelli, L. Neuder, W. Raphael, and J. Pursley. 2011. Effects of cloprostenol sodium at final prostaglandin F 2α of Ovsynch on complete luteolysis and pregnancy per artificial insemination in lactating dairy cows. Journal of dairy science 94:2815-2824.

Matsuyama, S., T. Kojima, S. Kato, and K. Kimura. 2012. Relationship between quantity of IFNT estimated by IFN-stimulated gene expression in peripheral blood mononuclear cells and bovine embryonic mortality after AI or ET. Reprod Biol Endocrinol 10:21.

McCracken, J. A., E. E. Custer, and J. C. Lamsa. 1999. Luteolysis: a neuroendocrine-mediated event. Physiological reviews 79:263-323.

Meyer, M., P. Hansen, W. Thatcher, M. Drost, L. Badinga, R. Roberts, J. Li, T. Ott, and F. Bazer. 1995. Extension of corpus luteum lifespan and reduction of uterine secretion of prostaglandin F 2α of cows in response to recombinant interferon-τ. Journal of dairy science 78:1921-1931.

Mialon, M., S. Camous, G. Renand, J. Martal, and F. Menissier. 1993. Peripheral concentrations of a 60-kDa pregnancy serum protein during gestation and after calving and in relationship to embryonic mortality in cattle. Reproduction Nutrition Development 33:269-282.

Moreira, F., R. De la Sota, T. Diaz, and W. Thatcher. 2000. Effect of day of the estrous cycle at the initiation of a timed artificial insemination protocol on reproductive responses in dairy heifers. Journal of animal science 78:1568-1576.

Moreira, F., C. Orlandi, C. Risco, R. Mattos, F. Lopes, and W. Thatcher. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. Journal of Dairy Science 84:1646-1659.

Niswender, G. D., J. L. Juengel, P. J. Silva, M. K. Rollyson, and E. W. McIntush. 2000. Mechanisms controlling the function and life span of the corpus luteum. Physiological reviews 80:1-29.

Ott, T., G. Van Heeke, C. Hostetler, T. Schalue, J. Olmsted, H. Johnson, and F. Bazer. 1993. Intrauterine injection of recombinant ovine interferon-tau extends the interestrous interval in sheep. Theriogenology 40:757-769.

Padmanabhan, V., D. Battaglia, M. B. Brown, F. J. Karsch, J. S. Lee, W. Pan, D. J. Phillips, and J. Van Cleeff. 2002. Neuroendocrine control of follicle-stimulating hormone (FSH) secretion: II. Is follistatin-induced suppression of FSH secretion mediated via changes in activin availability and does it involve changes in gonadotropin-releasing hormone secretion? Biology of reproduction 66:1395-1402.

Patel, O. V., O. Yamada, K. Kizaki, T. Takahashi, K. Imai, and K. Hashizume. 2004. Quantitative analysis throughout pregnancy of placentomal and interplacentomal expression of pregnancy-associated glycoproteins-1 and-9 in the cow. Molecular reproduction and development 67:257-263.

Pieterse, M., O. Szenci, A. Willemse, C. Bajcsy, S. Dieleman, and M. Taverne. 1990. Early pregnancy diagnosis in cattle by means of linear-array real-time ultrasound scanning of the uterus and a qualitative and quantitative milk progesterone test. Theriogenology 33:697-707.

Pinedo, P., A. De Vries, and D. Webb. 2010. Dynamics of culling risk with disposal codes reported by Dairy Herd Improvement dairy herds. Journal of dairy science 93:2250-2261.

Pursley, J., M. Mee, and M. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF 2α and GnRH. Theriogenology 44:915-923.

Pursley, J., M. Wiltbank, J. Stevenson, J. Ottobre, H. Garverick, and L. Anderson. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus. Journal of Dairy Science 80:295-300.

Rajakoski, E. 1960. The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical, and left-right variations. Acta endocrinologica 34:S7-S68.

Rantala, M., O. Peltoniemi, T. Katila, and J. Taponen. 2009. Effect of GnRH dose on occurrence of short oestrous cycles and LH response in cyclic dairy heifers. Reproduction in domestic animals 44:647-652.

Revah, I. and W. Butler. 1996. Prolonged dominance of follicles and reduced viability of bovine oocytes. Journal of reproduction and fertility 106:39-47.

Rivera, F. A., L. G. Mendonça, G. Lopes, J. E. Santos, R. V. Perez, M. Amstalden, A. Correa-Calderón, and R. C. Chebel. 2011. Reduced progesterone concentration during growth of the first follicular wave affects embryo quality but has no effect on embryo survival post transfer in lactating dairy cows. Reproduction 141:333-342.

Roberts, J. S., J. A. McCracken, J. E. Gavagan, and M. S. Soloff. 1976. Oxytocin-Stimulated Release of Prostaglandin F2α from Ovine Endometrium in Vitro: Correlation with Estrous Cycle and Oxytocin-Receptor Binding1 1. Endocrinology 99:1107-1114.

Roberts, R. M., Y. Chen, T. Ezashi, and A. M. Walker. 2008. Interferons and the maternal–conceptus dialog in mammals. Pages 170-177 in Proc. Seminars in cell & developmental biology. Elsevier.

Robinson, R., M. Fray, D. Wathes, G. Lamming, and G. Mann. 2006. In vivo expression of interferon tau mRNA by the embryonic trophoblast and uterine concentrations of interferon tau protein during early pregnancy in the cow. Molecular reproduction and development 73:470-474.

Robinson, R., G. Mann, G. Lamming, and D. Wathes. 1999. The effect of pregnancy on the expression of uterine oxytocin, oestrogen and progesterone receptors during early pregnancy in the cow. Journal of Endocrinology 160:21-33.

Robinson, R., G. Mann, G. Lamming, and D. Wathes. 2001. Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows. Reproduction 122:965-979.

Romano, J. E. and J. E. Larson. 2010. Accuracy of pregnancy specific protein-B test for early pregnancy diagnosis in dairy cattle. Theriogenology 74:932-939.

Sangsritavong, S., D. Combs, R. Sartori, L. Armentano, and M. Wiltbank. 2002. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17β in dairy cattle. Journal of dairy science 85:2831-2842.

Santos, J., C. Narciso, F. Rivera, W. Thatcher, and R. Chebel. 2010. Effect of reducing the period of follicle dominance in a timed artificial insemination protocol on reproduction of dairy cows. Journal of dairy science 93:2976-2988.

Santos, J., W. Thatcher, R. Chebel, R. Cerri, and K. Galvao. 2004. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. Animal Reproduction Science 82:513-535.

Sartori, R., J. Haughian, R. Shaver, G. Rosa, and M. Wiltbank. 2004. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. Journal of dairy science 87:905-920.

Sartori, R., R. Sartor-Bergfelt, S. Mertens, J. Guenther, J. Parrish, and M. Wiltbank. 2002. Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. Journal of Dairy Science 85:2803-2812.

Sasser, R., J. Crock, and C. Ruder-Montgomery. 1988. Characteristics of pregnancy-specific protein B in cattle. Journal of reproduction and fertility. Supplement 37:109-113.

Sasser, R. G., C. A. Ruder, K. A. Ivani, J. E. Butler, and W. C. Hamilton. 1986. Detection of pregnancy by radioimmunoassay of a novel pregnancy-specific protein in serum of cows and a profile of serum concentrations during gestation. Biology of Reproduction 35:936-942.

Scott, B. D. 2016. Incorporation of dairy farm survey data and epidemiological patterns for agent-based simulation modeling of dairy herd dynamics. MS Thesis. Cornell University.

Silva, E., R. Sterry, D. Kolb, N. Mathialagan, M. McGrath, J. Ballam, and P. Fricke. 2007. Accuracy of a pregnancy-associated glycoprotein ELISA to determine pregnancy status of lactating dairy cows twenty-seven days after timed artificial insemination. Journal of dairy science 90:4612-4622.

Sirois, J. and J. Fortune. 1988. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biology of reproduction 39:308-317.

Skarzynski, D. J. and K. Okuda. 1999. Sensitivity of bovine corpora lutea to prostaglandin F2α is dependent on progesterone, oxytocin, and prostaglandins. Biology of reproduction 60:1292-1298.

Souza, A., H. Ayres, R. Ferreira, and M. Wiltbank. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology 70:208-215.

Spencer, T. E., W. C. Becker, P. George, M. A. Mirando, T. F. Ogle, and F. W. Bazer. 1995. Ovine interferon-tau regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone. Biology of reproduction 53:732-745.

Sterry, R., M. Welle, and P. Fricke. 2006. Effect of interval from timed artificial insemination to initiation of resynchronization of ovulation on fertility of lactating dairy cows. Journal of dairy science 89:2099-2109.

Stevenson, J., S. Hill, R. Nebel, and J. DeJarnette. 2014. Ovulation timing and conception risk after automated activity monitoring in lactating dairy cows. Journal of dairy science 97:4296-4308.

Stevenson, J. and G. Lamb. 2016. Contrasting effects of progesterone on fertility of dairy and beef cows. Journal of dairy science 99:5951-5964.

Stevenson, J. and S. Pulley. 2016. Feedback effects of estradiol and progesterone on ovulation and fertility of dairy cows after gonadotropin-releasing hormone-induced release of luteinizing hormone. Journal of dairy science 99:3003-3015.

Stocco, D. M. and B. J. Clark. 1996. Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. Biochemical pharmacology 51:197-205.

Szenci, O., J.-F. Beckers, P. Humblot, J. Sulon, G. Sasser, M. Taverne, J. Varga, R. Baltusen, and G. Schekk. 1998. Comparison of ultrasonography, bovine pregnancy-specific protein B, and bovine pregnancy-associated glycoprotein 1 tests for pregnancy detection in dairy cows. Theriogenology 50:77-88.

Szenci, O., J.-F. Beckers, J. Sulon, M. Bevers, L. Börzsönyi, L. Fodor, F. Kovacs, and M. Taverne. 2003. Effect of induction of late embryonic mortality on plasma profiles of pregnancy associated glycoprotein 1 in heifers. The Veterinary Journal 165:307-313.

Szenci, O., P. Humblot, J. Beckers, G. Sasser, J. Sulon, R. Baltusen, J. Varga, C. Á. Bajcsy, and M. Taverne. 2000. Plasma profiles of progesterone and conceptus proteins in cows with spontaneous embryonic/fetal mortality as diagnosed by ultrasonography. The Veterinary Journal 159:287-290.

Tsai, S.-J., K. Kot, O. Ginther, and M. C. Wiltbank. 2001. Temporal gene expression in bovine corpora lutea after treatment with PGF2alpha based on serial biopsies in vivo. Reproduction 121:905-913.

Valenza, A., J. Giordano, G. Lopes, L. Vincenti, M. Amundson, and P. Fricke. 2012. Assessment of an accelerometer system for detection of estrus and treatment with gonadotropin-releasing hormone at the time of insemination in lactating dairy cows. Journal of dairy science 95:7115-7127.

Vasconcelos, J., R. Silcox, G. Rosa, J. Pursley, and M. Wiltbank. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology 52:1067-1078.

Waldmann, A., O. Reksen, K. Landsverk, E. Kommisrud, E. Dahl, A. Refsdal, and E. Ropstad. 2001. Progesterone concentrations in milk fat at first insemination—effects on non-return and repeat-breeding. Animal reproduction science 65:33-41.

Wathes, D. and M. Hamon. 1993. Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrous cycle and early pregnancy of the ewe. Journal of Endocrinology 138:479-NP.

Wathes, D. and G. Lamming. 1994. The oxytocin receptor, luteolysis and the maintenance of pregnancy. Journal of reproduction and fertility. Supplement 49:53-67.

Welt, C., Y. Sidis, H. Keutmann, and A. Schneyer. 2002. Activins, inhibins, and follistatins: from endocrinology to signaling. A paradigm for the new millennium. Experimental biology and medicine 227:724-752.

Whitlock, B. K. and H. S. Maxwell. 2008. Pregnancy-associated glycoproteins and pregnancy wastage in cattle. Theriogenology 70:550-559.

Wiltbank, M. C., G. M. Baez, F. Cochrane, R. V. Barletta, C. R. Trayford, and R. T. Joseph. 2015. Effect of a second treatment with prostaglandin F2α during the Ovsynch protocol on luteolysis and pregnancy in dairy cows. Journal of dairy science 98:8644-8654.

Wiltbank, M. C. and J. R. Pursley. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. Theriogenology 81:170-185.

Wooding, F. 1982. The role of the binucleate cell in ruminant placental structure. Journal of reproduction and fertility. Supplement 31:31-39.

Xie, S., J. Green, J. B. Bixby, B. Szafranska, J. C. DeMartini, S. Hecht, and R. M. Roberts. 1997. The diversity and evolutionary relationships of the pregnancy-associated glycoproteins, an aspartic proteinase subfamily consisting of many trophoblast-expressed genes. Proceedings of the National Academy of Sciences 94:12809-12816.

Xie, S., B. G. Low, R. J. Nagel, K. K. Kramer, R. V. Anthony, A. P. Zoli, J.-F. Beckers, and R. M. Roberts. 1991. Identification of the major pregnancy-specific antigens of cattle and sheep as inactive members of the aspartic proteinase family. Proceedings of the National Academy of Sciences 88:10247-10251.

Zoli, A. P., L. A. Guilbault, P. Delahaut, W. B. Ortiz, and J.-F. Beckers. 1992. Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application for pregnancy diagnosis. Biology of Reproduction 46:83-92.

CHAPTER II

EMBRYO MORTALITY AROUND THE PERIOD OF MAINTENANCE OF THE CORPUS LUTEUM CAUSES ALTERATIONS TO THE OVARIAN FUNCTION OF LACTATING DAIRY COWS

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ABSTRACT

Objectives were to identify cows with embryo mortality (EM) around the period of corpus luteum (CL) maintenance by interferon tau (IFNT) and characterize ovarian function in cows that underwent EM. Lactating Holstein cows received AI (Day = 0) with semen or extender only. From Day 14 to 42 transrectal ultrasonography was performed daily to monitor ovarian dynamics and uterine contents whereas blood was collected every 48 h to determine *ISG15* and *MX2* mRNA abundance in blood mononuclear cells (Day 14 to 22 only) and determination of hormone concentrations. Cows were classified in the following reproductive status groups: cyclic (inseminated with extender; n = 15), pregnant (embryo present on Day 42; n = 23), no-embryo (n = 23), and EM (n = 14). Embryo mortality was defined as the presence of an embryo based on ISG mRNA abundance and concentrations of pregnancy-specific protein B (PSPB) above specific cutoff points but no embryo visualized by ultrasonography. Within the EM group, early EM (up to Day 22) was when ISG fold changes were above specific cutoff points from Day 18 to 22 and PSPB below 0.7 ng/mL on and after Day 24, whereas late EM (after Day 22) was when PSPB was above 0.7 ng/mL on or after Day 24 regardless of ISG expression. This experiment

provided evidence that the combination of ISG expression patterns and PSPB concentrations is a reasonable method to determine EM around the period of CL maintenance by IFNT as cows with evidence of EM had patterns of ISG expression more similar to pregnant than cyclic cows or cows with no-embryo. Within the EM group, only cows with late EM had delayed luteal regression and longer interovulatory intervals. No major alterations in follicular function were observed after the onset of luteolysis. Our results suggest that embryo development needs to continue beyond 22 days after AI to effectively prevent luteolysis and extend the luteal phase.

INTRODUCTION

Pregnancy loss during the embryonic period of gestation [up to 42 days after AI; (Hubbert, 1972)] is a major cause of poor reproductive performance of lactating dairy cow (Santos et al., 2004; Moore et al., 2005). Unquestionably, the greatest detriment to cow reproductive performance is embryo loss. Nevertheless, cows that lose their pregnancy may also exhibit alterations in ovarian function leading to delayed estrus expression and re-insemination (Bartolome et al., 2005; Chebel et al., 2006; Remnant et al., 2015). Pregnancy loss may also be responsible, at least in part, for the poor response to synchronization of estrus and ovulation treatments used for previously inseminated non-pregnant cows (Giordano et al., 2012b; Lopes et al., 2013; Bruno et al., 2014; Giordano et al., 2015).

Timing of embryo demise is critical because embryo losses before the beginning of the period of corpus luteum (CL) maintenance by interferon tau (IFNT; ~14 to 16 days of gestation; (Farin et al., 1990; Robinson et al., 2006)) are not expected to affect ovarian function. By contrast, cows that experience embryo mortality (EM) during or after the period of CL maintenance might present extended luteal phases and interovulatory intervals due to abrogation

of the luteolytic mechanism by conceptus-derived IFNT (Knickerbocker et al., 1986; Helmer et al., 1989; Meyer et al., 1995). Because of the lack of reliable methods to detect the presence of embryos around the period of CL maintenance or simple models to mimic the process in-vivo, the complex relationship between the occurrence and timing of EM around the period of CL maintenance and ovarian function has not been characterized in detail. Previous studies estimated embryo presence and subsequent demise based on the interval from AI to a next estrus, circulating concentrations of progesterone (P4), circulating concentrations of pregnancy associated glycoproteins (PAG) (Humblot, 2001; Grimard et al., 2006; Gábor et al., 2007; Breukelman et al., 2012), or combinations thereof. Although these studies provided useful insights into the relationship between EM and ovarian function, embryo losses might have been either overestimated or underestimated. For example, return to estrus and circulating concentrations of P4 overestimate the occurrence of EM by assuming that lack of estrus or luteal regression is exclusively due to the presence of an embryo during the period of CL maintenance. By contrast, using PAG to determine the presence of embryos during the period of CL maintenance may underestimate pregnancy losses because the first detectable rise in PAG in blood with current laboratory assays typically occurs 24 days following AI (Green et al., 2005; Giordano et al., 2012a). Experimental models of induction of CL maintenance (Northey and French, 1980; Heyman et al., 1984; Meyer et al., 1995) using conceptus-derived proteins (i.e., IFNT) and tissues, or removal of the conceptus from the uterus at different stages of pregnancy mimic to some extent the effect of EM, however, these models may not fully replicate the physiological conditions of naturally occurring EM.

More recently, changes in the pattern of gene expression in circulating immune cells in response to IFNT have been used to determine the presence of an embryo during the period of

CL maintenance in cattle (Han et al., 2006; Gifford et al., 2007; Stevenson et al., 2007; Green et al., 2010). Cows confirmed pregnant through visualization of a viable embryo (32 to 45 days after AI) presented increased expression of interferon stimulated genes (ISG) such as the myxovirus resistance proteins 1 and 2 (*MX1* and *MX2*), 2'-5'-oligoadenylate synthetase-1, and interferon stimulated gene 15 (*ISG15*) in peripheral blood leucocytes when compared with non-pregnant or cyclic cows (Han et al., 2006; Gifford et al., 2007; Stevenson et al., 2007; Green et al., 2010; Pugliesi et al., 2014). Expression of ISG was characterized by maximum mRNA expression from approximately 18 to 22 days after AI (Han et al., 2006; Pugliesi et al., 2014) coincident with a period of known high IFNT release in cattle (Farin et al., 1990; Robinson et al., 2006). Therefore, this novel approach may be used in combination with other methods such as measurement of PAG in circulation as an experimental tool to provide evidence of the presence of an embryo during the period of CL maintenance in cattle by IFNT.

Our hypotheses were that: 1) coupling the determination of ISG expression in peripheral blood mononuclear cells (PBMC) and measurement of circulating concentrations of PSPB (marker of a suite of PAG) during the period of CL maintenance by IFNT will facilitate the identification of cows with EM and 2) EM during or immediately after the period of CL maintenance will extend the CL lifespan and affect follicular wave dynamics and ovulation. Thus, our first objective was to identify lactating dairy cows that underwent EM through determination of changes in ISG expression in PBMC and PSPB concentrations in blood. Thereafter, our objective was to characterize in detail the patterns of CL growth and regression and follicular dynamics in cows that underwent EM as determined by ISG and PSPB. Defining the extent of EM around the period of CL maintenance and its impact on ovarian function is a

necessary first step for the development of novel strategies to improve reproductive efficiency of lactating dairy cows.

MATERIALS AND METHODS

This research was conducted from June to December of 2013 at the Dairy Unit of the Cornell University Ruminant Center (Harford, NY). All procedures were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at Cornell University and complied with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (McGlone, 2010).

Cows were housed in freestall barns and were fed a total mixed ration once a day with ad libitum access to feed and water. The diet was formulated to meet or exceed nutrient requirements for lactating dairy cows producing 45 kg of milk per day (Cornell Net Carbohydrate and Protein System). Cows were milked three times per day at approximately 8 h intervals and received recombinant bovine somatotropin (500 mg of Sometribove zinc; Posilac, Elanco Animal Health, Indianapolis, IN) at 14 days intervals beginning approximately at 60 days in milk until dry off.

Lactating primiparous (n = 37) and multiparous (n = 58) Holstein cows were synchronized with the Presynch-Ovsynch protocol [prostaglandin F2α (PGF2α)-14 days-PGF2α-12 days- gonadotropin releasing hormone (GnRH)-7 days-PGF2α-56 h-GnRH-16 h-AI; (Moreira et al., 2001)) starting on average at 33 days in milk (range = 24 to 43 days in milk) to receive timed AI (TAI). At TAI, cows were stratified by parity and randomly assigned to be sham inseminated with semen extender only or inseminated with regular semen (henceforth referred to as semen) from seven bulls of proven fertility in a ratio of approximately 1 sham insemination to

4 inseminations with semen. In total, 20 cows were inseminated with extender only and 75 cows with semen. Straws for sham inseminations and straws containing sperm were packaged by the same laboratory (Genex CRI, Ithaca, NY) and using the same extender used for frozen semen preparation. Inseminations were performed by one professional AI technician from Genex. Research personnel were blind to treatments during data collection.

Determination of ovarian function and pregnancy status

Transrectal ultrasonography (TUS) of the ovaries was conducted using a portable ultrasound machine (Ibex Pro, Ibex, Loveland, CO). In all enrolled cows, TUS was performed on Days 0, 2, and 7 (TAI = 0) to assess the response to the synchronization of ovulation protocol. Cows that did not ovulate after TAI were excluded from the experiment. Thereafter, TUS of the ovaries was performed daily from Day 14 to 42 (Figure 2.1).

At each examination, the position and diameter of follicles greater than 4 mm and corpora lutea was recorded on an ovarian map. All measurements were performed during each examination using the ultrasound machine internal digital calipers. Follicle and CL diameter was the average of the two longest perpendicular measurements. Follicle growth was determined by dividing the total growth (mm) between two specific time points by the number of days between the time points. Ovulation was defined as the disappearance of at least one follicle ≥10 mm between two consecutive examinations and confirmed by the presence of a new CL 7 days after presumptive ovulation (ovulation was not confirmed 7 days after presumptive ovulation in two cows because they ovulated 40 days after AI) on the ovary where the putative ovulatory follicle was observed. Changes in steroid hormone concentrations [estradiol (E2) and progesterone (P4)] were also used to confirm ovulation.

The uterus of each cow was evaluated daily to determine the presence or absence of uterine fluid, placental membranes, and an embryo.

Blood sample collection

Blood was collected into 8-mL heparinized evacuated tubes (Vacutainer, BD, Franklin Lakes, NJ) via puncture of the coccygeal vein or artery on Days 0, 2, and 7 to determine circulating concentrations of P4. These samples were used to verify the response to the synchronization of ovulation protocol. Thereafter, from Day 14 to 42 after TAI, blood collection was performed every 48 h to determine circulating concentrations of P4, E2, and PSPB. Samples were immediately placed into crushed ice and centrifuged at 1700 x g for 20 minutes at 4°C within 2 h of collection. Plasma was harvested and transferred into two different storage tubes and stored at -20°C until assays were performed.

On Days 14, 16, 18, 20, and 22 a blood sample was collected using cell preparation tubes (CPT tubes, Vacutainer, BD, Franklin Lakes, NJ) to harvest PBMC (lymphocytes and monocytes) for subsequent RNA extraction. Tubes were kept at room temperature and within 1 to 2 h of collection, samples were centrifuged for 30 minutes at 1500 x g at room temperature. Cells were re-suspended in the plasma layer and transferred into 15 mL conical tubes and centrifuged for 15 minutes at 500 x g at 4°C. After centrifugation, the supernatant was removed and the PBMC pellet was lysed with 200 µl Trizol (Invitrogen, Carlsbad, CA) and frozen at -80°C until assayed.

Determination of progesterone concentrations

Progesterone concentrations in plasma were determined in duplicate using a commercial solid-phase, no-extraction RIA (Coat-a-count; Diagnostic Products Corp., Los Angeles, CA). To assess precision of the assay, control samples with high (7.4 ng/mL) and low (0.5 ng/mL) concentrations of P4 were included at the beginning and end of each assay (n = 15 assays). Average detection limit for the P4 assay was 0.03 ng/mL. Average intra-assay CV for the high-concentration sample for the 15 assays performed was 8%, whereas the inter-assay CV was 11%. For the low-concentration sample the average intra-assay CV for the 15 assays was 14%, whereas the inter-assay CV was 21%.

Determination of pregnancy-specific protein B concentrations

Concentrations of PSPB in plasma were determined using a commercially available sandwich ELISA assay (BioPRYN, BioTracking LLC, Moscow, ID). Samples were shipped to BioTracking LLC and the assay performed in their laboratory. A standard curve was added to the commercial assay to determine PSPB concentrations. Six PSPB standards (0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 ng/mL) and a blank were used for analysis. A curve was fitted to the standards on each plate using a linear least squares regression. The resulting standard curve was used to determine the concentration of PSPB in each sample. Samples were run in duplicate in two separate assays. For assay validation, two control samples (high and low) containing PSPB were added to all plates in triplicate to calculate inter- and intra-assay CV. Average intra-assay CV for the high-concentration sample was 2.4%, whereas the inter-assay CV was 3.6%. For the low-concentration sample the intra-assay CV was 2.9% whereas the inter-assay CV was 4.1%. Average detection limit for the assay was 0.3 ng/mL.

Determination of estradiol concentrations

Estradiol concentrations in plasma were determined by a double antibody RIA (MaiaZen Estradiol R-FA-120, Zen Tech SA, Belgium) after benzene:toluene extraction. Average detection limit was 0.3 pg/mL. To assess the precision of the assays (n = 10 assays), a control sample (6.5 pg/mL) was added in quadruplicate (2 x 100 µL and 2 x 200 µL) at the beginning and end of each assay. The average intra-assay CV was 16% and the inter-assay CV was 17%.

RNA extraction and cDNA Synthesis

After thawing PBMC, RNA was extracted and purified following the manufacturer instructions (Trizol, Invitrogen, Carlsbad, CA). Concentration and quality of RNA were assessed using a Nanodrop (ND 1000 spectrophotometer, version 3.8.1,Thermoscientific, Wilmington, DE, USA) and with an Experion automated electrophoresis system with the Experion RNA StdSens kit (Bio-Rad, Hercules, CA) and the Experion software version 3.0 (Agilent technologies, Wilmington, DE) following manufacturer's instructions. Because heparin was used during sample collection, heparinase (H2519, Sigma Aldrich, St. Louis, MO) treatment was used to avoid interference with the assay. Briefly, samples were incubated with 1 U of heparinase I/µg of RNA, 40 U of RNasin (PRN2511, Promega Corporation, Madison, WI) and buffer containing 5 mM Tris buffer at a pH of 7.5, with 1 mM CaCl2 for 2 h at 25°C. This was followed by DNase treatment which was carried out using RQ1 RNase free DNase kit (M6101; Promega Corporation, Madison, WI). Briefly, 1 µg of RNA was incubated with 1 µL of RQ1 DNase 10X Reaction Buffer and 1 µL of RQ1 RNase-Free DNase in 8 µL total volume at 37°C for 30 min. One µL of DNase stop solution was added and incubated for 10 min at 65°C. Complementary DNA synthesis was done using a Dynamo cDNA synthesis kit (F470L; Thermo Scientific,

Waltham, MA). A master mix made up of 10 µL RT buffer, 1 µL of random hexamer primers, 2 µL of M-MuLV RNase H^+ reverse transcriptase with 1 µg of DNAse treated RNA sample was incubated for 10 min at 25°C for primer extension, 30 min incubation at 37°C for cDNA synthesis and 5 min at 85 °C for reaction termination. As a negative control for the reaction, four reaction master mixes were prepared without M-MuLV RNase H^+ reverse transcriptase and served as no RT controls for our PCR assays. After cDNA synthesis, samples were stored at -20 °C.

Quantitative Real Time PCR for ISG15 and MX2 gene expression

Except for annealing temperature (Table 1), all reactions were completed as follows. Real time PCR was done using SensiMix SYBR No-ROX Kit (QT650-05, Bioline USA, Taunton, MA). Fifteen microliters of a master mix containing 10 µL of SYBR Green, 0.33 µL (15 µM) of reverse primer, 0.33 µL (15 µM) of forward primer and 4.34 µL of nuclease free water was added into each well. Five µL of cDNA template (previously diluted 1:5 with nuclease free water) was added (each sample was ran in duplicate) and the last two wells were loaded with no RT and water only controls. The following run parameters were used: denaturation at 95°C for 5 min followed by 40 cycles of amplification: denaturation at 95 C for 30 sec, annealing (see Table 2.1 for details about each gene) for 45 sec, extension 72°C for 1 min. This was followed by a final extension step of 72°C for 5 min, followed by melting curve analysis. Duplicate samples that differed by more than 0.5 CT were re-analyzed. Standard curves consisting of 12 log dilutions of PCR amplified template were used to validate the assays. Standard curves exhibited Ct values ranging from 5 to 35 and samples with Ct values beyond the range were excluded. Standard curves had slopes between -3.2 and -3.3, R^2 values of 0.98 or greater and

efficiencies between 100 and 105%. Signal intensity was captured and analyzed using ABI 7500 Fast real time PCR machine (Applied Biosystems, Life Technologies). Ribosomal protein L19 (*RPL19*) was used as a reference gene and ΔCt's, ΔΔCt's and fold changes for *ISG15* and *MX2* were calculated as described in Livak and Schmittgen (2001) using each cow's day 14 as reference. The reference gene was chosen based on the absence of effects of treatments on the abundance of its mRNA and similar cDNA amplification efficiencies between target genes and the reference gene calculated here and elsewhere (Ribeiro et al., 2014).

Creation of reproductive status groups

Cows that received sham insemination were included in the cyclic reproductive status group (CY) whereas cows with a viable pregnancy based on TUS examination at the end of the study, 42 days after AI, were included in the pregnant reproductive status group (PG). On the other hand, cows that received AI with semen and had no evidence of an embryo during the experimental period were considered as nonpregnant (NP). Further, NP cows were retrospectively classified into a no-embryo (NE) or embryo mortality (EM) group. Cows were included in the EM reproductive status group if there was evidence of the presence of an embryo as determined by *ISG15* and *MX2* gene expression in PBMC and/or PSPB concentrations in plasma but no viable embryo was observed with TUS during the experimental period. Conversely, cows in the NE group did not have evidence of the presence of an embryo based on ISG expression and plasma concentrations of PSPB.

Because specific criteria to determine the presence of an embryo based on ISG expression have not been established, we used Receiver Operating Characteristic (ROC) curve analysis to determine cutoff values for *ISG15* and *MX2* fold changes that provided evidence of

the presence of an embryo from Day 18 to 22. Cyclic cows were used to provide data for negative outcomes because these cows did not have an embryo present any time after AI. Cows in the PG group were used to provide data for positive outcomes because all had an embryo present from Day 18 to 22. Cows in the NP reproductive status group were not included in the ROC analysis because they constituted the group of interest for determination of the presence or absence of an embryo from Day 18 to 22 based on ISG. The ROC analysis option of MedCalc (version 12.5.0.0; MedCalc Software bvba, Ostend, Belgium) was used to create the ROC curves. For each day of interest (i.e., 18, 20, and 22 days after AI) data for fold change (compared to Day 14) for CY (negative outcome) and PG (positive outcome) cows were used to determine the minimum fold change that indicated with the greatest combined sensitivity and specificity the presence of an embryo (positive outcome).

After a fold change cutoff value for each gene (i.e., *ISG15* and *MX2*) for each day was determined, cows with no evidence of a viable embryo based on TUS (i.e., NP reproductive status group) were re-classified into the NE or EM reproductive groups as follows. Cows assigned to the EM group had a fold change above the cutoff value determined by ROC for either *ISG15*, *MX2*, or both on Days 18, 20, and 22 (e.g., *ISG15* above cutoff on Days 18 and 20 but not on Day 22 and *MX2* above cutoff on Day 22 but not on Days 18 and 20. At least one of the two genes was above the cutoff for each one of the days of interest). Evidence of embryo presence was also based on circulating concentrations of PSPB $\geq$ 0.7 ng/mL on or after Day 24. The cutoff selected for PSPB was based on the mean PSPB concentration for PG cows on Day 24 (0.7 ng/mL). Cows in the NE reproductive status group did not have a fold change above the cutoff value determined by ROC during any day or no more than two days during the days of interest (e.g., no *ISG15* or *MX2* above the cutoff for any day or *ISG15* above the cutoff on Days

18 and 20 but both ISG below the cutoff on Day 22) and PSPB concentrations did not reach 0.7 ng/mL on and after Day 24.

Further, to explore the effect of EM at different time points on ovarian function, cows in the EM group were re-classified based on ISG expression and PSPB concentrations in two additional reproductive status groups: 1) Early embryo mortality (EEM): evidence of embryo presence based on ISG expression but PSPB concentrations below 0.7 ng/mL on and after Day 24 and 2) Late embryo mortality (LEM): circulating concentrations of PSPB above 0.7 ng/mL for at least one day on or after Day 24.

Statistical analysis

This research was conducted as a mix of a completely randomized design (i.e., random assignment of cows to insemination with extender only or semen) and a prospective cohort study (i.e., determination of EM and ovarian function from 14 to 42 days after AI).

ISG expression differences between groups: Two separate analyses were conducted to evaluate ISG expression differences between reproductive status groups. For the first analysis, reproductive status groups (i.e., CY, NP, and PG) created based on the initial TUS diagnosis were included. The second analysis included reproductive status groups (CY, NE, EM, and PG) created based on ISG expression and PSPB concentrations as described. Differences in ΔΔCts for *ISG15* and *MX2* for the different reproductive status groups were analyzed by ANOVA using the MIXED procedure of SAS (Version 9.4; SAS Institute, Cary, NC). Normality of data for ΔΔCts was assessed using graphical methods (histogram and Q-Q plot) generated with the UNIVARIATE procedure of SAS and the residual option of the MIXED procedure. Reproductive status group (either CY, NP, and PG or CY, NE, EM, and PG), day after AI, the

reproductive status group by day interaction, and parity were included in the model as fixed effects, whereas cow nested within reproductive status group was included as a random effect in all models.

Ovarian dynamics: Two analyses were conducted to evaluate ovarian function. The first analysis included cows in the CY, NE, and EM reproductive status groups, whereas the second analysis included cows in the CY, NE, EEM, and LEM reproductive status groups. Binomial outcomes (proportion of cows with complete luteal regression and proportion of cows ovulating) were analyzed using Fisher's exact test through the FREQ procedure of SAS because some reproductive status groups had less than five positive outcomes and for some reproductive status groups 100% of the cows had a positive outcome. Mean separation analysis was not conducted for binomial outcomes because there was an insufficient number of observations for certain subgroups for a meaningful comparison. Continuous variables (days to onset of luteal regression, days to complete luteal regression, days to ovulation, days from onset of luteal regression to ovulation, days from complete luteal regression to ovulation, maximum ovulatory follicle diameter, ovulatory follicle diameter before ovulation, ovulatory follicle growth rate to maximum diameter and ovulatory follicle growth rate before ovulation) were analyzed by ANOVA using the MIXED procedure of SAS. The day of the onset of luteal regression was defined as the day before P4 concentrations dropped 50% below the maximum concentration observed and continued decreasing in the subsequent samples. The day of complete luteal regression was the day after ovulation at which P4 concentration was below 0.5 ng/mL. For cows that underwent complete luteal regression, E2 concentrations were evaluated after normalization to the day of the onset of luteal regression and the day of complete luteal regression, whereas for cows that presented complete luteal regression and ovulated, E2 concentrations were normalized

to the day of ovulation. Because data for E2 concentrations were not normally distributed, values were log transformed before conducting the analysis and back transformed for reporting. In all cases, models included reproductive status group as a fixed effect, whereas cow nested within reproductive status group was included as a random effect. When the effect of a categorical explanatory variable was significant, the Least Significant Difference *post-hoc* mean separation test was used to determine differences between means.

Survival analysis for time to complete luteal regression and ovulation after AI was conducted using Cox's proportional hazards regression with the PHREG procedure of SAS with reproductive status group as fixed effect. Kaplan-Meier survival curves were generated to illustrate the hazard of complete luteal regression and ovulation after AI using the Survival Analysis option of MedCalc.

All values for quantitative outcomes are reported as Least Square Means (± SEM) unless otherwise stated whereas for binomial outcomes proportions generated using the FREQ procedure of SAS are reported. All explanatory variables and their interactions were considered significant if $P < 0.05$ whereas P-values between 0.05 and 0.10 were considered a tendency.

RESULTS

Classification of cows into reproductive status groups and incidence of embryo mortality

Figure 2.2 includes a graphical depiction of the reproductive status groups generated, the criteria used to generate the groups, and the number of cows included in the final analysis presented herein. Seventeen cows were removed from the study because they failed to ovulate after AI (n = 8), presented clinical health disorders (n = 3), or left the herd before completion of the study (n = 6). After removal of these cows, data were available from 15 cows that received

sham insemination (CY reproductive status group; 7 primiparous and 8 multiparous) and 63 cows inseminated with semen (27 primiparous and 36 multiparous). Data for ISG was not available for one cow in the CY reproductive status group because the RNA sample was lost during processing, however, data for this cow were included in the analysis of ovarian function.

Within the group of cows inseminated with semen and available for analysis, 40 cows were considered NP and 23 cows were PG (36.5% pregnancy per AI) based on the initial classification through TUS 42 days after AI. Three cows in the NP reproductive status group that completed the experimental period presented an embryo with a heartbeat 30 to 35 days after AI but the embryo died before Day 42. Including these cows in the PG rather than NP reproductive status group would have resulted in 41.3% pregnancy per AI. These three cows were excluded from all analyses because their pregnancy reached a further stage of development (embryo with a heartbeat visualized by TUS) than in cows classified into the embryo mortality group based on ISG expression and concentrations of PSPB only (*described below*).

After re-classification of cows based on ISG expression fold changes and plasma PSPB concentrations, 23 cows remained in the NE reproductive status group and 14 cows were included in the EM reproductive status group. Based on this classification, 62% (37/60) of the cows inseminated with semen had evidence of the presence of an embryo. Of these cows, 38% (14/37) underwent EM after Day 18 based on the criteria used to define EM in the current study. Cut off criteria, sensitivity, specificity and area under the curve for gene expression fold change to determine the presence of an embryo on Days 18, 20, and 22 are shown in Table 2.2. Day 18 was selected as the earliest day to define a criterion because it was the first day after AI when mRNA relative abundance was different between CY and PG groups (*data presented in Interferon Stimulated Genes Expression section*). Individual cow ISG fold changes for Days 18,

20, and 22, and PSPB concentrations on Days 24 and 26 for cows in the EM reproductive status group are shown in Supplementary Table 2.1.

Furthermore, among cows with evidence of EM, 50% (n = 7) underwent EEM and 50% (n = 7) LEM. Figure 2.3 includes individual circulating concentrations for PSPB for cows in the LEM group and the mean ± 3 SD for cows in the PG group from 20 to 42 days after AI. Figure 2.4 includes the relative abundance of mRNA for *ISG15* and *MX2*, and circulating concentrations of P4, E2, and PSPB for two representative cows in the EEM (Figure 2.4A and 4B) and two cows in the LEM reproductive status group, respectively (Figure 2.4C and 4D).

Interferon Stimulated Genes expression

The mRNA abundance for *ISG15* and *MX2* for cows in the CY, PG, and NP groups was affected by reproductive status group ($P < 0.001$) and the reproductive status group by day interaction ($P < 0.001$; Figure 2.5A and 5B). Days after AI tended to affect ($P = 0.10$) *ISG15* mRNA abundance and affected ($P < 0.001$) *MX2* expression. Based on mean separation analysis, PG cows had greater *ISG15* and *MX2* mRNA abundance than CY and NP cows on Days 18, 20, and 22. There was no effect of parity ($P > 0.10$) on *ISG15* or *MX2* mRNA abundance.

When cows in the NP reproductive status group were subdivided in the NE and EM groups, mRNA abundance for *ISG15* and *MX2* were affected by reproductive status group ($P < 0.001$), day (*ISG15*: $P = 0.04$; *MX2*: $P < 0.001$), and the reproductive status group by day interaction ($P < 0.001$; Figure 2.6A and 6B). Cows in the PG and EM groups had greater *ISG15* and *MX2* mRNA abundance on Days 18 and 20 than cows in the CY and NE groups. On Day 22, cows in the PG group had the greatest ISG mRNA abundance of all reproductive status groups. Cows in the EM group had greater *ISG15* and *MX2* mRNA abundance than cows in the NE and

CY groups (Figure 2.6A and 6B). There was no effect of parity (P > 0.1) for *ISG15* or *MX2* mRNA abundance.

Ovarian function in cows that underwent embryo mortality

Luteal regression: The proportion of cows that underwent complete luteal regression by Day 42 was affected (P < 0.01) by reproductive status group because fewer cows in the EM group presented complete luteal regression than cows in the CY and NE groups (Table 2.3). The timing of the onset of luteal regression (P < 0.001) and complete luteal regression (P < 0.01) was also affected by reproductive status group because cows in the EM group had more days to the onset and to complete luteal regression than cows in the CY and NE groups (Table 2.3). The hazard of complete luteal regression from Day 14 to 42 was greater (P < 0.01; Figure 2.7A) for cows in the CY than the EM group (HR 5.6; 95% CI: 2.1 – 15.8) and for cows in the NE than the EM group (HR 3.5; 95% CI: 1.4 – 8.6). Conversely, the hazard of complete luteal regression did not differ between the CY and NE groups (HR 1.6; 95% CI: 0.8 – 3.1).

Ovulation and follicular parameters: The proportion of cows ovulating within 42 days after AI was less (P = 0.02) for cows in the EM than the CY and NE reproductive status groups (Table 2.3). Furthermore, days from AI to ovulation were affected by reproductive status group (P = 0.03) such that cows in the EM and NE groups had more days from AI to ovulation than cows in the CY reproductive status group (Table 2.3). Mean days from the onset of luteal regression and complete luteal regression to ovulation were not affected by reproductive status group (Table 2.3). The hazard of ovulation from 14 to 42 days after AI was greater (P < 0.01; Figure 2.7B) for cows in the CY (HR 4.6, 95% CI: 1.9 – 11.9) and the NE groups (HR 2.5, 95% CI: 1.1 – 5.7)

than for cows in the EM group. Conversely, the hazard of ovulation did not differ for the CY and NE reproductive status groups (HR 1.9, 95% CI: 0.9 – 3.7).

Maximum diameter of the ovulatory follicle, ovulatory follicle diameter before ovulation, growth rate to maximum size, and ovulatory follicle growth rate for the last 5 days before ovulation (cows that failed to ovulate not included) were not affected by reproductive status group (Table 2.3).

Estradiol concentrations: Estradiol concentrations were affected ($P < 0.001$) by day relative to the onset of luteal regression, complete luteal regression, and ovulation. There was no effect ($P > 0.10$) of reproductive status group or the reproductive status group by day interaction on E2 concentrations relative to the onset and to complete luteal regression (Figure 2.8A and 8B). Conversely, concentrations of E2 tended ($P = 0.05$) to differ for cows in the CY, NE, and EM reproductive status groups when concentrations were normalized to the day of ovulation (Figure 2.8C).

Ovarian function in cows with early versus late embryo mortality

Luteal regression: The proportion of cows undergoing complete luteal regression by 42 days after AI was affected by reproductive status group ($P < 0.01$; Table 2.4). Cows undergoing LEM had more days to the onset of luteal regression ($P < 0.001$) and complete luteal regression ($P < 0.001$) than cows in the CY, NE, and EEM reproductive status group (Table 2.4). The hazard of complete luteal regression was greater ($P < 0.01$; Figure 2.9A) for cows in the CY than the LEM group (HR = 11.7; 95% CI: 3.2 – 42.7), for cows in the NE than the LEM group (HR = 7.2; 95% CI: 2.1 – 25.1), and for cows in the EEM than the LEM group (HR = 4.3; 95% CI: 1.2 – 15.9).

The hazard of complete luteal regression was similar for cows in the CY and NE (HR = 1.6; 95% CI: 0.8 – 3.1), CY and EEM (HR = 2.7; 95% CI: 0.9 – 7.6), and EEM and NE reproductive status groups (HR = 0.6; 95% CI: 0.2 – 1.6).

Ovulation and follicular parameters: The proportion of cows ovulating within 42 days after AI was affected by reproductive status group ($P < 0.01$; Table 2.4) which also affected the timing of ovulation after AI ($P < 0.001$). Cows in the LEM group had the longest interval to ovulation whereas cows in the EEM group had similar interval to ovulation than cows in the NE and CY groups (Table 2.4). Cows in the NE group had more days to ovulation than cows in the CY group. The hazard of ovulation was greater ($P < 0.01$; Figure 2.9B) for cows in the CY than cows in the LEM group (HR = 13.6; 95% CI: 2.9 – 61.9), for cows in the NE than the LEM group (HR = 7.2; 95% CI: 1.7 – 31.4), and for cows in the EEM than the LEM group (HR = 7.3; 95% CI: 1.5 – 36.4). The hazard of ovulation was similar for cows in the CY and NE group (HR = 1.9; 95% CI: 0.9 – 3.7), CY and EEM (HR = 1.8; 95% CI: 0.7 – 4.9), and for cows in the EEM and NE groups (HR = 1.0; 95% CI: 0.4 – 2.5).

Table 2.1. Gene, primer orientation and sequence (5′ to 3′), annealing temperatures and NCBI sequences for RPL19, *MX2*, and *ISG15* primers used in quantitative real time PCR.

Gene	Primer	Sequence (5′ to 3′)	°C[1]	NCBI sequence
RPL19	forward primer	ATCGATCGCCACATGTATCA	60	NM_001040516.1
	reverse primer	GCGTGCTTCCTTGGTCTTAG		
MX2	forward primer	CTTCAGAGACGCCTCAGTCG	57	NM_173941.2
	reverse primer	TGAAGCAGCCAGGAATAGTG		
ISG15	forward primer	GGTATCCGAGCTGAAGCAGTT	60	NM_174366.1
	reverse primer	ACCTCCCTGCTGTCAAGGT		

[1]Annealing temperature

Table 2.2. Cut off value (criterion), sensitivity, specificity, and area under the curve with 95% confidence intervals obtained by Receiver Operating Characteristic curve analysis for *ISG15* and *MX2*. The criterion for fold change determined by Receiver Operating Characteristic curve analysis predicted with the highest combined sensitivity and specificity the presence of an embryo for each day of interest.

Day after AI	*ISG15*					*MX2*				
	Criterion	Se[1] (%)	Sp[2] (%)	AUC[3]	95% CI	Criterion	Se[1] (%)	Sp[2] (%)	AUC[3]	95% CI
18	2.1	73.7	91.7	0.84	0.67 - 0.95	1.5	94.7	94.9	0.90	0.78 - 0.98
20	2.6	75.0	92.3	0.89	0.74 - 0.97	1.9	95.0	100.0	0.99	0.87 - 1.00
22	1.6	100.0	76.9	0.90	0.75 - 0.98	1.4	95.0	76.9	0.93	0.78 - 0.99

[1]Se = sensitivity
[2]Sp = specificity
[3]AUC = area under the curve

Table 2.3. Ovarian function parameters for cows in the Cyclic, Embryo mortality, and No embryo reproductive status group.

	Reproductive status group			
	Cyclic	No Embryo	Embryo mortality	P-value
Complete luteal regression % (n/n)	100 (15/15)	100 (23/23)	71 (10/14)	< 0.01
Days to onset of luteal regression	18.5 ± 0.5[a]	19.8 ± 0.5[a]	27.2 ± 2.8[b]	< 0.001
Days to complete luteal regression[1]	20.7 ± 0.6[a]	22.4 ± 0.5[a]	26.8 ± 2.7[b]	< 0.01
Ovulation % (n/n)	93 (14/15)	91 (21/23)	57 (8/14)	0.02
Days AI to ovulation	23.1 ± 0.5[a]	25.7 ± 0.6[b]	27.5 ± 2.4[b]	0.03
Days from onset of luteal regression to ovulation	4.9 ± 0.5	6.2 ± 0.6	6.4 ± 1.0	0.27
Days from complete luteal regression to ovulation	2.9 ± 0.4	3.5 ± 0.6	3.5 ± 1.1	0.27
Maximum diameter ovulatory follicle (mm)	20.5 ± 0.6	19.0 ± 0.6	20.3 ± 1.2	0.20
Diameter before Ovulation (mm)	20.5 ± 0.6	18.8 ± 0.6	20.3 ± 1.0	0.16
Growth rate to maximum diameter (mm/d)	1.5 ± 0.2	1.4 ± 0.1	1.3 ± 0.2	0.22
Growth rate to ovulatory diameter (mm/d)	1.4 ± 0.2	1.3 ± 0.1	1.1 ± 0.2	0.46

[1]Two cows in the embryo mortality group initiated luteolysis but P4 did not fall below 0.5 ng/mL by Day 42.
Different letters in a same row indicates P < 0.05.

Table 2.4. Ovarian function parameters for cows in the Cyclic, Early embryo mortality, Late embryo mortality, and No embryo reproductive status groups as determined by transrectal ultrasonography, pregnancy-specific protein B, and ISG expression in peripheral blood mononuclear cells.

	Reproductive status group				
	Cyclic	No Embryo	EEM[1]	LEM[2]	P-value
Cows with complete luteal regression, % (n/n)	100 (15/15)	100 (23/23)	86 (6/7)	57 (4/7)	< 0.01
Days to onset of luteal regression	18.5 ± 0.5[a]	19.8 ± 0.5[a]	22.1 ± 3.4[a]	34.2 ± 2.9[b]	< 0.001
Days to complete luteal regression[3]	20.7 ± 0.6[a]	22.4 ± 0.5[a]	21.1 ± 1.5[a]	35.0 ± 3.1[b]	< 0.001
Cows ovulating, % (n/n)	93 (14/15)	91 (21/23)	86 (6/7)	29 (2/7)	< 0.01
Interval from AI to Ovulation, (d)	23.1 ± 0.5[a]	25.7 ± 0.6[b]	24.5 ± 1.6[ab]	36.5 ± 3.5[c]	< 0.001
Days from onset of luteal regression to ovulation	4.9 ± 0.5	6.2 ± 0.6	5.5 ± 1.5	9.0 ± 0	0.13
Days from complete luteal regression to ovulation	2.9 ± 0.4	3.5 ± 0.6	3.2 ± 1.4	4.5 ± 2.5	0.13
Maximum diameter ovulatory follicle (mm)	20.5 ± 0.6	19.0 ± 0.6	19.5 ± 1.2	22.8 ± 2.7	0.14
Diameter before Ovulation (mm)	20.5 ± 0.6	18.8 ± 0.6	19.5 ± 1.2	21.5 ± 1.5	0.22
Growth rate to maximum diameter (mm/d)	1.5 ± 0.2	1.4 ± 0.1	1.3 ± 0.2	1.1 ± 0.5	0.71
Growth rate to ovulatory diameter (mm/d)	1.4 ± 0.2	1.3 ± 0.1	1.3 ± 0.2	0.5 ± 0.1	0.20

[1]Early embryo mortality.
[2]Late embryo mortality.
[3]One cow in the Early embryo mortality group and one cow in the Late embryo mortality group initiated luteolysis but progesterone did not fall below 0.5 ng/mL by Day 42.
Different letters in a same row indicates P< 0.05.

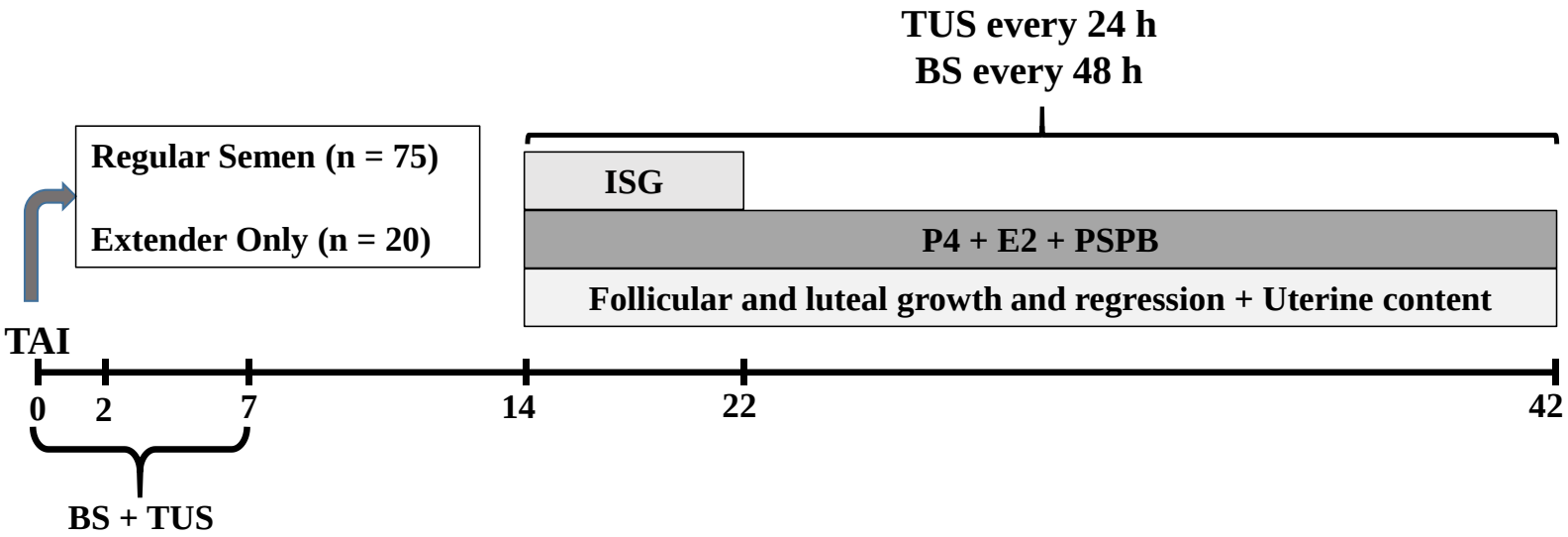

Figure 2.1. Schematic representation of experimental procedures. Ninety five lactating Holstein cows were synchronized using the Presynch-Ovsynch protocol and at the time of AI (Day = 0) randomly assigned to receive AI with extender only or regular semen in a ratio of approximately 1 to 5. Transrectal ovarian ultrasonography was conducted and blood samples were collected at the time of AI and then 2 and 7 days after AI to verify ovulation and formation of a functional corpus luteum (progesterone ≥1 ng/mL) based on circulating concentrations of progesterone and estradiol. From Day 14 to 42, transrectal ultrasonography was conducted every 24 h to record the presence and size (diameter) of all ovarian structures and evaluate uterine content. In addition, blood samples were collected every 48 h for determination of circulating concentrations of progesterone, estradiol, and pregnancy-specific protein B. From Day 14 to 22, another blood sample was collected every 48 h to harvest peripheral blood mononuclear cells for mRNA extraction and determination of interferon stimulated genes (*ISG15* and *MX2*) expression. TAI = timed AI, BS = blood sample, TUS = transrectal ultrasonography, ISG = interferon stimulated genes, P4 = progesterone, E2 = estradiol, PSPB = pregnancy-specific protein B.

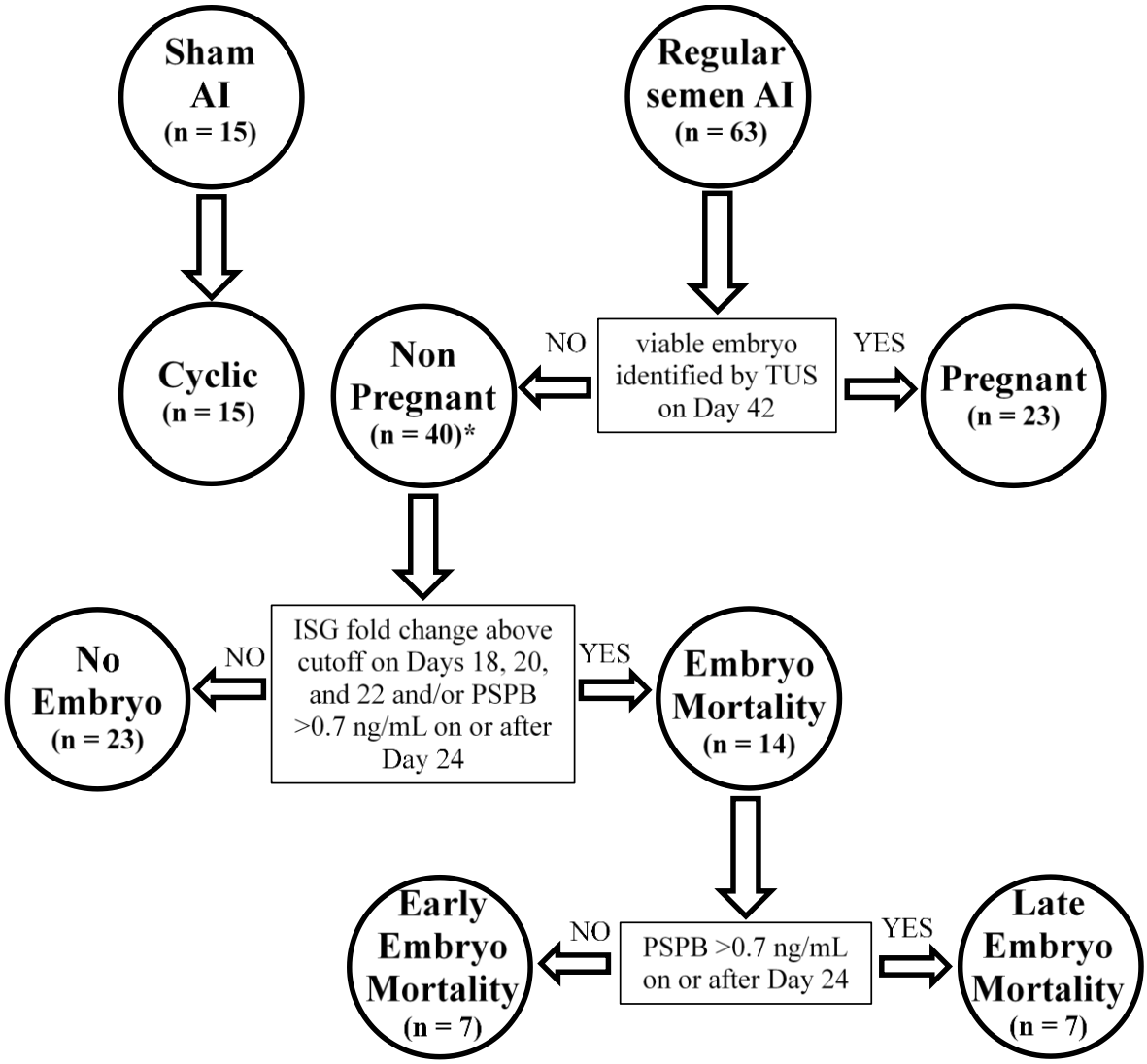

Figure 2.2. Schematic representation of classification of cows to reproductive status groups for this study. Cows inseminated with semen extender only (sham AI) were assigned to the Cyclic group. Cows that received AI with regular semen were classified into a Pregnant or Nonpregnant group based on the visualization or not of a viable embryo with transrectal ultrasonography on Day 42. Within the Nonpregnant group, cows were assigned to the Embryo mortality or No embryo group based on ISG expression cutoff thresholds established using ROC analysis and plasma concentrations of pregnancy-specific protein B. Cows in the Embryo mortality group were further subdivided into an Early embryo mortality (ISG mRNA relative abundance above established cutoff on Days 18, 20, and 22 after AI and PSPB < 0.7 ng/mL on and after Day 24) and Late embryo mortality group (PSPB > 0.7 ng/mL on or after Day 24). *Three cows considered nonpregnant on Day 42 based on transrectal ultrasonography presented an embryo with a heartbeat 30 to 35 days after AI. These cows were excluded from all analyses because the pregnancy reached a further stage of development (embryo with a heartbeat visualized by ultrasonography) than for cows classified into the Embryo mortality group. TUS = transrectal ultrasonography, ISG = interferon stimulated genes, PSPB = pregnancy-specific protein B.

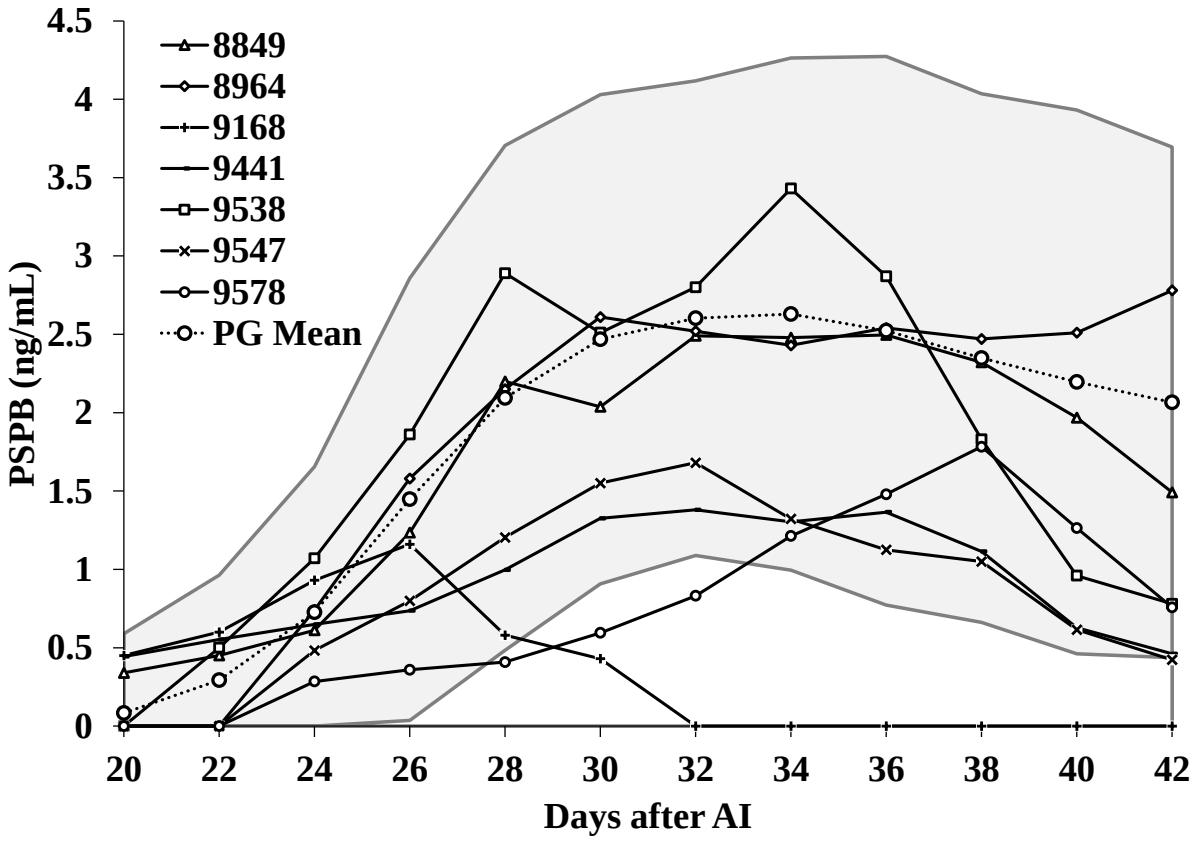

Figure 2.3. Individual profiles for circulating concentrations of pregnancy-specific protein B from Day 20 to 42 for cows that underwent late embryo mortality and mean concentrations (dashed line with open circles) ± 3 SD (shaded area) for pregnant cows. PSPB = pregnancy-specific protein B.

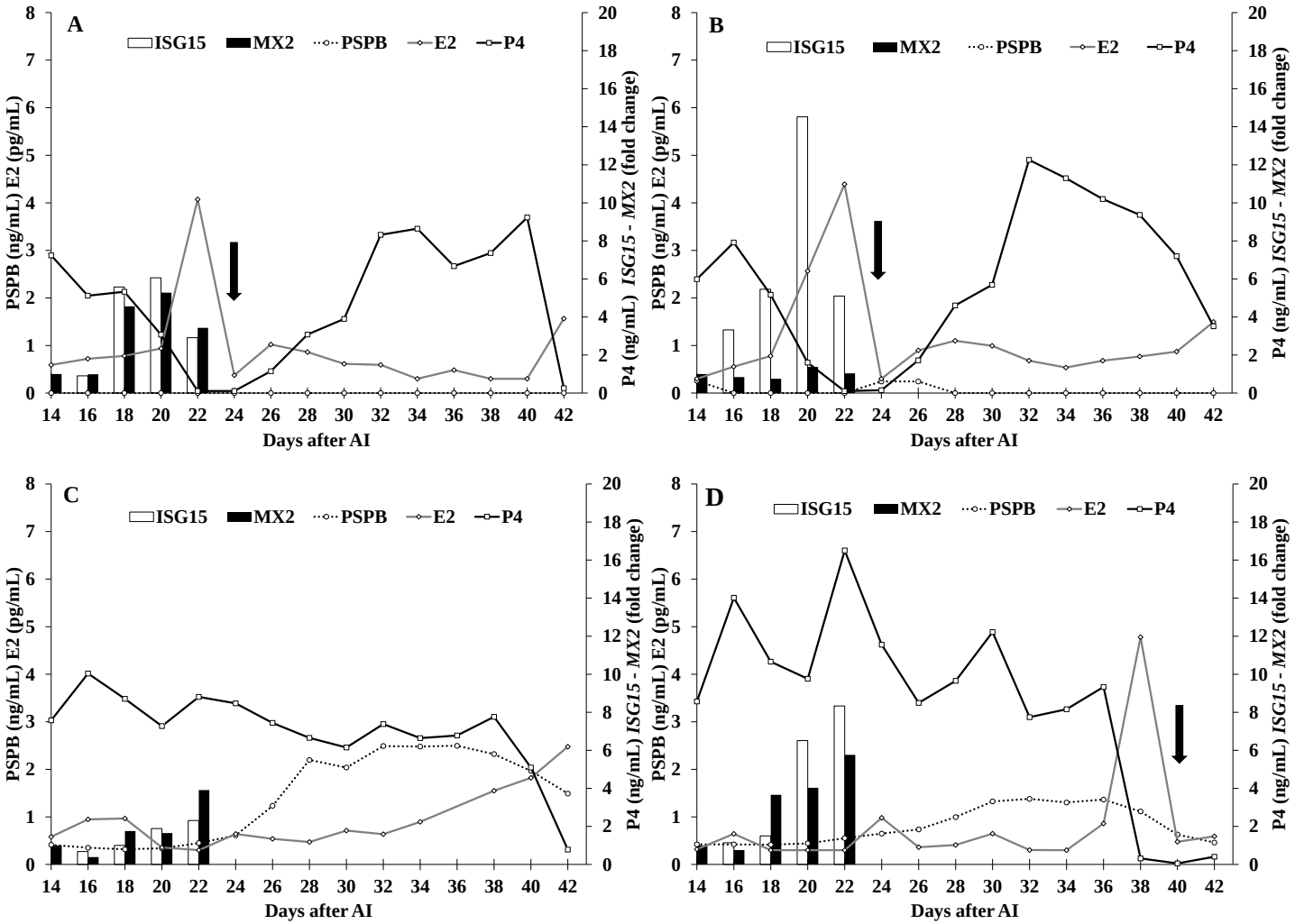

Figure 2.4. Individual progesterone, estradiol, and pregnancy-specific protein B profiles from Day 14 to 42, and *ISG15* and *MX2* mRNA expression fold change from Day 14 to 22 for two representative cows in the Early embryo mortality (A and B) and Late embryo mortality reproductive status groups (C and D), respectively. P4 = progesterone, E2 = estradiol, and PSPB = pregnancy-specific protein B. Black arrows indicate day of ovulation when it corresponds.

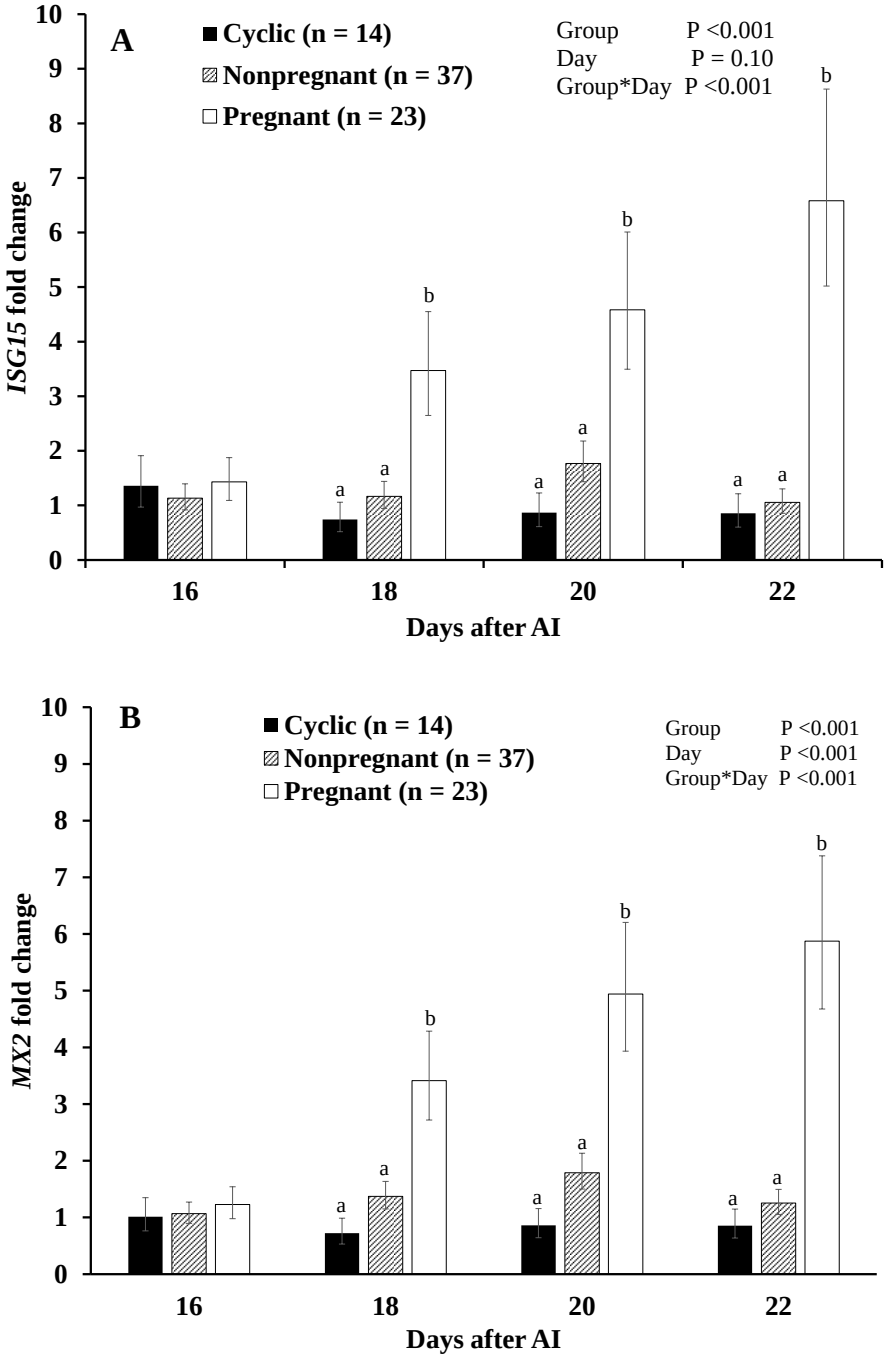

Figure 2.5. Relative mRNA abundance for *ISG15* (A) and *MX2* (B) for Cyclic, Nonpregnant and Pregnant cows as diagnosed by transrectal ultrasonography. Fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method using Day 14 as reference. Error bars were calculated as $2^{-(\Delta\Delta Ct \pm 1\ SEM)}$. Different letters within the same day indicate $P < 0.05$.

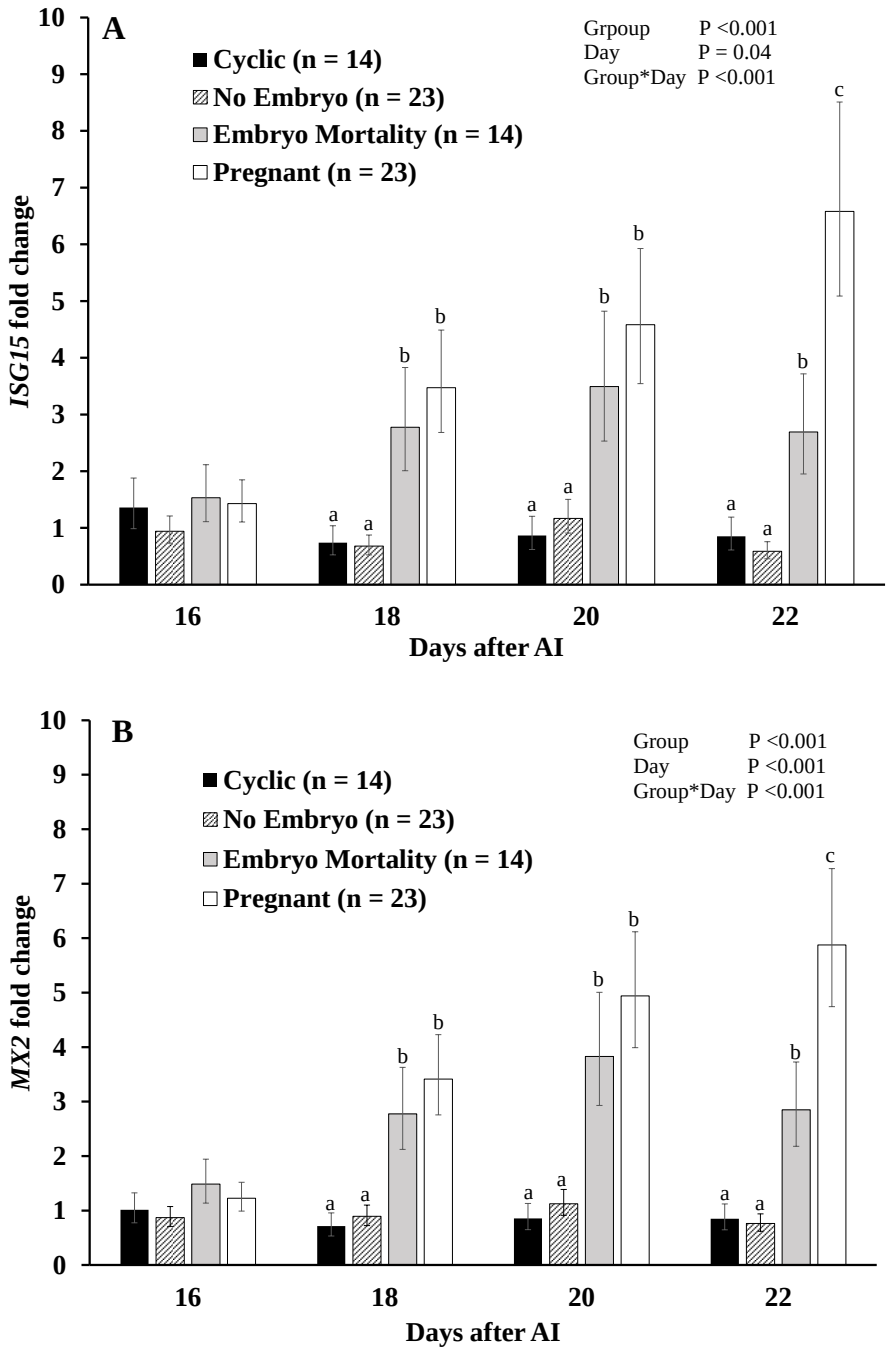

Figure 2.6. Relative mRNA abundance for *ISG15* (A) and *MX2* (B) for cows in the Cyclic, No embryo, Embryo mortality, and Pregnant reproductive status groups. Embryo mortality was determined based on ISG expression cutoff thresholds established using ROC analysis and plasma concentrations of pregnancy-specific protein B. Fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method using Day 14 as reference. Error bars were calculated as $2^{-(\Delta\Delta Ct \pm 1\ SEM)}$. Different letters within the same day indicate $P < 0.05$. ISG = interferon stimulated genes.

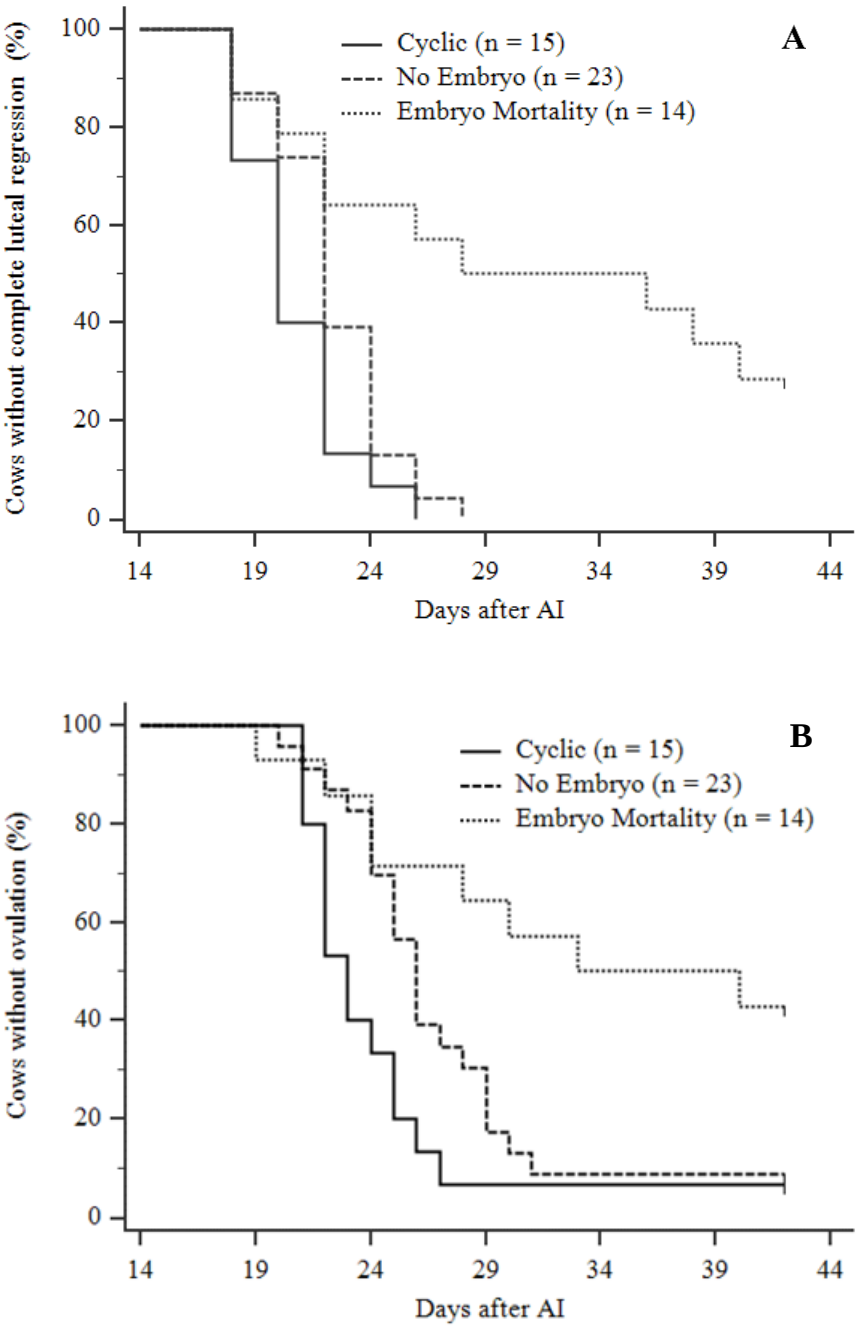

Figure 2.7. Kaplan-Meier survival curves for time to complete luteal regression (progesterone <0.5 ng/mL) (A) and ovulation (B) up to Day 42 for cows in the Cyclic (CY), No embryo (NE), and Embryo mortality (EM) reproductive status groups. The hazard of complete luteal regression was affected by ($P < 0.01$) reproductive status group. The hazard of complete luteal regression for the different groups compared were: CY vs EM: HR = 5.6 (95% CI: 2.1 – 15.8), NE vs EM: HR = 3.5 (95% CI: 1.4 – 8.6), CY vs NE: HR = 1.6 (95% CI: 0.8 – 3.1). The hazard of ovulation was affected by ($P < 0.01$) reproductive status group. Ovulation after AI: CY vs EM: HR = 4.6 (95% CI: 1.9 – 11.9), NE vs EM: HR = 2.5 (95% CI: 1.1 – 5.7), CY vs NE: HR = 1.9 (95% CI: 0.9 – 3.7).

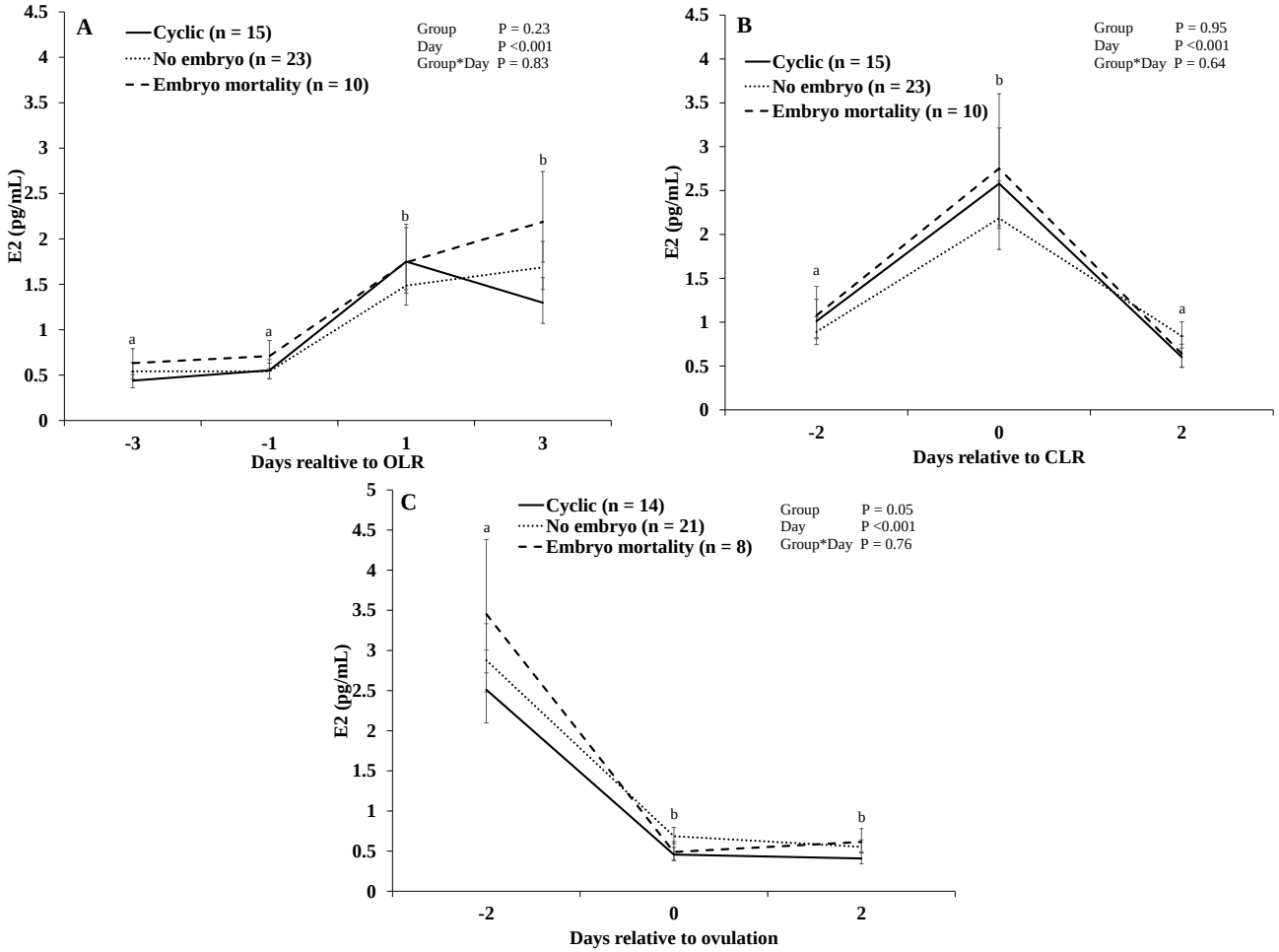

Figure 2.8. Concentrations of estradiol (back transformed data of the log of the LSM ± 95% CI) for cows in the Cyclic, No embryo, and Embryo mortality reproductive status group relative to the onset of luteal regression (A), complete luteal regression (B) and ovulation (C). Only cows that underwent the onset of luteal regression, complete luteal regression, and ovulation were included in the respective analyses. Different letters across days indicate $P < 0.05$. E2 = estradiol.

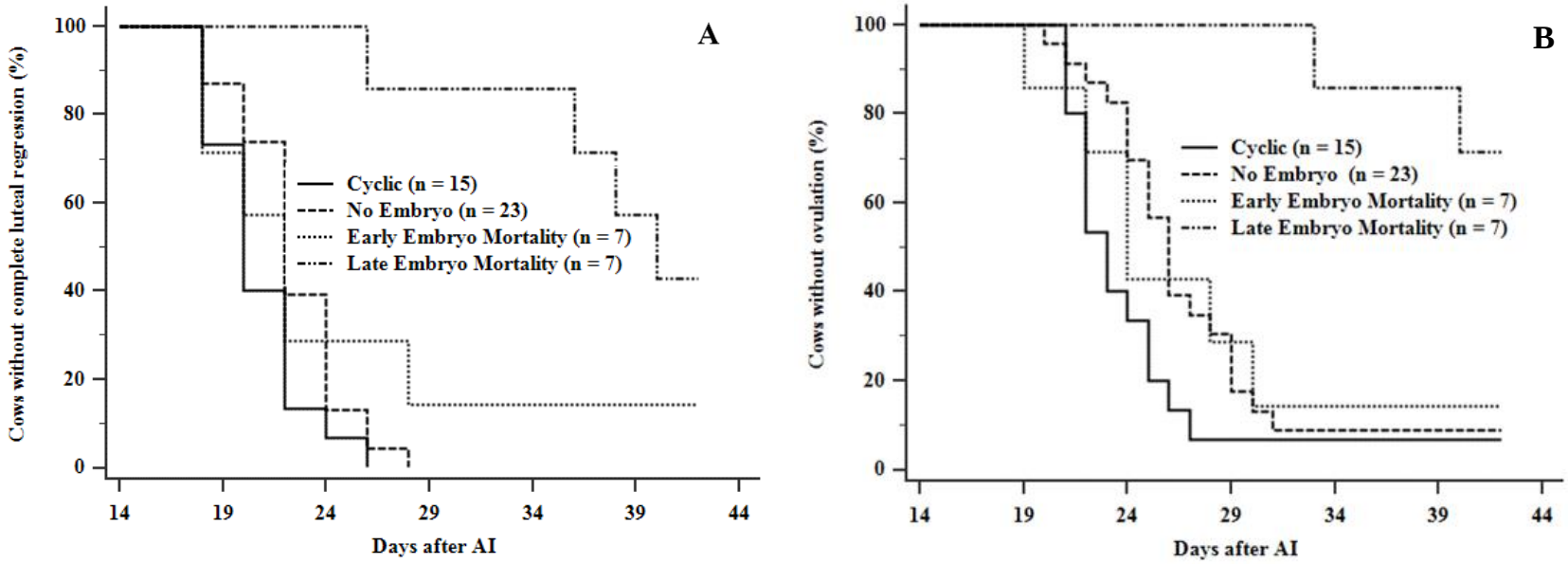

Figure 2.9. Kaplan-Meier survival curves for time to complete luteal regression (progesterone < 0.5 ng/mL) (A) and ovulation (B) up to Day 42 for cows in the Cyclic (CY), No embryo (NE), Early embryo mortality (EEM), and Late embryo mortality (LEM) reproductive status groups. The hazard of complete luteal regression was affected by (P <0.01) reproductive status group. The hazard of complete luteal regression for the different groups compared were: CY vs LEM (HR = 11.7; 95% CI: 3.2 – 42.7), NE vs LEM (HR = 7.2; 95% CI: 2.1 – 25.1), EEM vs LEM (HR = 4.3; 95% CI: 1.2 – 15.9), CY vs NE (HR = 1.6; 95% CI: 0.8 – 3.1), CY vs EEM (HR = 2.7; 95% CI: 0.9 – 7.6) and, EEM vs NE (HR = 0.6; 95% CI: 0.2 – 1.6). The hazard of ovulation was affected by (P <0.01) reproductive status group. The hazard of ovulation for the different groups compared were: CY vs LEM (HR = 13.6; 95% CI: 2.9 – 61.9), NE vs LEM (HR = 7.2; 95% CI: 1. 7 – 31.4), EEM vs LEM (HR = 7.3; 95% CI: 1.5 – 36.4), CY vs NE (HR = 1.9; 95% CI: 0.9 – 3.7), CY vs EEM (HR = 1.8; 95% CI: 0.7 – 4.9) and, EEM vs NE (HR = 1.0; 95% CI: 0.4 – 2.5).

DISCUSSION

A necessary first step to test the hypothesis that EM around the period of CL maintenance by IFNT alters ovarian function was to identify cows that underwent EM. In the current study we defined EM based on ISG expression in PBMC and circulating concentrations of PSPB (marker of a suite of PAG). Greater *ISG15* and *MX2* mRNA abundance for the PG than CY reproductive status group from 18 to 22 days after AI indicated that the presence of an embryo induced substantial changes in ISG mRNA relative abundance in PBMC during a period of expected IFNT secretion by the embryo and abrogation of luteolysis (Knickerbocker et al., 1986; Meyer et al., 1995; Robinson et al., 2006). On the other hand, the lack of difference in ISG mRNA expression for the CY and NP reproductive status groups suggested that cows classified as non-pregnant did not have an embryo present during the period of CL maintenance. Our observations for the PG and NP reproductive status groups are in agreement with previous studies that retrospectively classified cows based on pregnancy status determined by TUS at 32 to 45 days after AI (Han et al., 2006; Gifford et al., 2007; Green et al., 2010). Nevertheless, a major caveat of evaluating ISG expression retrospectively in cows diagnosed nonpregnant based on TUS several days after determination of ISG expression, is that embryo death from the time of ISG determination to the time of TUS cannot be determined. Because of this, we hypothesized that within the NP group it would be possible to identify cows with evidence of the presence and subsequent demise of an embryo through ISG expression and PSPB concentrations. In our experiment, the potential confounding effect of undetected EM for the determination of cutoff values for ISG was eliminated by including a group of cyclic cows not exposed to an embryo and a group of cows with a viable pregnancy up to 42 days after AI. Thus, in support of our hypothesis when cows assigned to the NP group were subdivided in the NE and EM groups,

cows in the EM group exhibited *ISG15* and *MX2* mRNA relative abundance similar to pregnant cows and greater than cows in the NE and CY reproductive status groups. In addition, the lower ISG mRNA relative abundance exhibited by cows in the EM than PG group on Day 22 was likely a reflection of poor embryo quality, delayed embryo development, embryo demise, or a combination of these for some cows in the EM group. In agreement, Ribeiro et al. (2016a) recently reported lower concentrations of IFNT in the uterine fluid of cows with embryos at earlier than later stages of development (ovoid < tubular < filamentous) 15 days after AI. On the other hand, the similar pattern of ISG expression for the NE and CY groups suggests that in cows from the NE group, no embryo developed up to the period of CL maintenance by IFNT or that embryo development did not involve substantial production of IFNT.

Based on our criteria to define the presence and subsequent demise of an embryo, 62% of the cows inseminated with semen (67% when including the three cows with EM >30 days after AI) presented evidence of an embryo around the period of CL maintenance by IFNT. Our results are in agreement with recent studies in which 66% [40/60; data combined from references (Mann and Lamming, 2001; Mann et al., 2006; Cerri et al., 2012)] of cows had an embryo at slaughter 16 to 17 days after AI and two recent studies in which 61% (134/220) of cows were considered pregnant based on detection of IFNT in the uterine fluid 15 days after AI (Ribeiro et al., 2016a; Ribeiro et al., 2016b). Likewise, our estimation of EM is in agreement with the results of Han et al. (2006) who reported that 33% (4/12) of lactating dairy cows considered pregnant based on their ISG15 mRNA profile were diagnosed nonpregnant by TUS 32 days after AI.

Unfortunately, the results of our study are not directly comparable to many of the previous reports which have estimated EM around the period of maintenance of the CL by IFNT because most studies based their estimation of EM on return to estrus, P4 profiles, PAG profiles,

or their combination (Humblot, 2001; Grimard et al., 2006; Gábor et al., 2007; Breukelman et al., 2012). Determination of EM based on P4 profiles after AI lacks specificity because it does not provide evidence of the presence of an embryo. The use of PAG provides direct evidence of the presence of an embryo but not all cows exhibit detectable concentrations during the early period of CL maintenance by IFNT with current laboratory assays (Green et al., 2005; Giordano et al., 2012a).

The criteria used in the current study to identify cows with EM also has weaknesses because it relied on indirect evidence of IFNT production by the embryo and the presence of an embryo was not confirmed by other methods. In addition, it may have underestimated or overestimated the presence of embryos based on ISG mRNA relative abundance because ISG expression may be affected by factors other than IFNT. For example, infectious processes are known to induce ISG expression (Hansen et al., 2010; Smirnova et al., 2012; Weiner et al., 2012). We attempted to minimize the potential confounding effect of infections on ISG expression by removing all cows that exhibited clinical signs of disease; however, it is possible that infections that did not cause clinical signs affected our results. Indeed, subclinical infections could explain, at least in part, why two cows in the CY group exhibited ISG mRNA relative abundance levels indicative of the presence of an embryo. On other hand, we attempted to minimize false positive outcomes and overestimating EM by defining the presence of an embryo based on ISG when cows presented fold changes for *ISG15* or *MX2* above the ROC cutoff point for three days rather than only one or two. In this regard, Han et al. (2006) reported greater predictive ability of pregnancy status in dairy cows when using *ISG15* mRNA levels for at least four consecutive days beginning 18 days after AI rather than on Day 18 only. However, this stringent method to classify cows resulted in the potential misclassification of three cows based

on ISG (no embryo present based on ISG) in the LEM group. Detailed examination of ISG expression for these cows revealed that two cows had *ISG15* or *MX2* fold changes above the respective cutoff for each gene for at least one day. The other cow presented ISG fold changes above the cutoff for two days. It is unclear at the moment why these cows did not exhibit ISG expression above the cutoff for each one of the days analyzed but had detectable PSPB concentrations. Interestingly, two of these cows were primiparous which in a previous study (Green et al., 2010) but not in ours, had greater ISG expression than multiparous cows at the same stage of pregnancy. It is possible that these cows had smaller embryos which produced less IFNT (Mann and Lamming, 2001; Liebig, 2015; Ribeiro et al., 2016a; Ribeiro et al., 2016b), and thereby stimulated less ISG mRNA expression in PBMC (Liebig, 2015). Indeed, a positive dose response relationship was documented after intrauterine infusion of IFNT (Matsuyama et al., 2012) or in vitro treatment (Shirasuna et al., 2012) for ISG expression in PBMC in cattle. Likewise, two cows in the PG reproductive status group also failed to present ISG above the cutoff during the three days (one cow had ISG expression over the threshold two days whereas the other one day only). In agreement, Han et al. (2006) reported that 22% of lactating dairy cows diagnosed pregnant by TUS 32 days after AI were incorrectly diagnosed nonpregnant based on serial determination of *ISG15* mRNA abundance. Thus, additional research is needed to better characterize the multiple factors other than IFNT that may induce ISG expression and the ISG response in PBMC to embryos of different sizes and stages of development in lactating dairy cows.

The most notable outcome of IFNT secretion by the bovine embryo is the prevention of luteolysis (Roberts et al., 1996; Spencer et al., 2006). Thus, we expected cows with EM during or immediately after the period of CL maintenance by IFNT to exhibit altered ovarian function; in

particular extended luteal phases and/or increased interovulatory intervals. In support of our hypothesis, cows with EM were less likely to undergo complete luteal regression, had delayed luteal regression, reduced ovulation rate, and longer interovulatory intervals. Moreover, we observed substantial variation for the timing of complete luteal regression among cows in the EM reproductive status group with a range of 18 to 40 days after AI and ~30% of the cows without complete luteal regression by the end of the observation period 42 days after AI. Our results are in agreement with previous studies which documented altered ovarian dynamics in cows with evidence of pregnancy loss based on PAG concentrations (Humblot, 2001; Grimard et al., 2006; Gábor et al., 2007; Breukelman et al., 2012), in cows that received intrauterine infusions of IFNT (Meyer et al., 1995), or in cows in which conceptus lysates were infused into the uterus before or during the expected time of CL maintenance by IFNT (Northey and French, 1980). To the best of our knowledge this is the first study to document alterations to ovarian dynamics in cows with putative EM based on ISG expression in PBMC and circulating PAG concentrations.

Cows with evidence of EM were separated further in an EEM and LEM groups because we speculated that ovarian function could be affected differently depending on the timing of EM. Based on the current understanding of ISG expression and PAG concentration patterns, the observation of PSPB concentrations similar to those of cows with a viable pregnancy was interpreted as an indication of further embryo development than when only ISG mRNA above the cutoff points selected was observed. For cows in the EEM reproductive status group it was assumed that an embryo survived to produce sufficient IFNT to induce ISG expression, however, the embryo died before producing detectable amounts of PSPB. Our observations for the EEM and LEM reproductive status groups offered interesting insights about the relationship between

EM and ovarian function. Only cows that suffered LEM were less likely to undergo complete luteal regression, have delayed luteal regression, and longer interovulatory intervals than cows in the CY and NE reproductive status groups, whereas cows in the EEM group exhibited patterns of CL regression and ovulation similar to those of cows in the NE and CY reproductive status groups. Our observations are in agreement with the traditional definition of EEM and LEM based on the timing of return to estrus after AI whereby, return to estrus before 24 days after AI is indicative of lack of fertilization or EEM, whereas return to estrus after 24 days after AI is indicative of LEM (Humblot, 2001). The traditional definition of EEM and LEM was based on the notion that extension of the interestrus interval beyond 24 days is due to embryo demise at least 16 days after ovulation when substantial IFNT production is initiated in cattle (Farin et al., 1990; Robinson et al., 2006). Our current results, however, suggest that embryo death from 16 to 22 days after AI does not necessarily extend the luteal phase. For cows in the EEM reproductive status group there was evidence of the presence of an embryo based on ISG mRNA from 18 to 22 days after AI but these cows completed luteal regression at a similar time as cows in the CY and NE reproductive status groups. A plausible explanation for these observations is that embryos from cows in the EEM group survived beyond Day 16 and reached a stage of development at which they produced sufficient IFNT to induce ISG expression but insufficient to prevent luteolysis. These embryos might have been developmentally impaired, and thereby of smaller size than expected during the period of CL maintenance by IFNT. Indeed, the presence of a smaller embryo was associated with lower IFNT concentrations in the uterine lumen (Mann and Lamming, 2001; Ribeiro et al., 2016a; Ribeiro et al., 2016b) and a greater 13, 14-dihydro-15-keto-prostaglandin F2alpha (a PGF2α metabolite) response to an oxytocin challenge (Mann and Lamming, 2001). Also, greater ISG mRNA abundance in PBMC was reported for cows that

received intrauterine infusion of greater doses (1,000 vs. 500 μg) of recombinant IFNT (Matsuyama et al., 2012). Despite these observations, we cannot speculate about the size or the capability of embryos to produce IFNT in cows with EEM because a quantitative relationship between ISG mRNA abundance in PBMC with embryo size and IFNT production in-vivo has not been determined for cows. Asynchrony between the actual time of IFNT production and the time at which IFNT was necessary to prevent luteolysis is another possible explanation for our observations. Indeed, for some cows, the onset of luteolysis was observed before Day 18 to 22 when ISG expression was evident (Figure 2.4A and 2.4B) suggesting that luteolysis began before the embryo was capable of producing sufficient IFNT to prevent it.

Based on the fact that in cows with evidence of EEM from 18 to 22 days after AI the luteal phase and interovulatory interval were not extended, we speculate that in lactating dairy cows normal conceptus development needs to continue beyond 22 days of gestation to effectively prevent luteolysis and extend the luteal phase substantially. Unfortunately, the exact relationship between days of exposure to IFNT or to an embryo in-vivo and the magnitude of the extension of the luteal phase is unknown. Notably, studies that intended to mimic the process of CL maintenance by IFNT through i.m. injections of IFN-α from Day 14 to 17 (Plante et al., 1984), uterine infusion of conceptus lysates twice daily from Day 14 to 18 (Northey and French, 1980), or embryo removal from Day 17 to 19 (Northey and French, 1980) extended the luteal phase for only 3 to 4 days. In contrast, the luteal phase was extended by 8 days in cows that received twice daily intrauterine infusions of IFNT from Day 14 to 24 (Meyer et al., 1995) and some cows did not present complete luteal regression by the end of the observation period 35 days after ovulation. An implication of our observations for the timing of EM and its relationship with ovarian function is that the duration of the luteal phase or the interovulatory interval may

not be accurate predictors of the occurrence and timing of EM because embryo demise during the period of CL maintenance by IFNT did not necessarily extend the luteal phase and the interovulatory interval. In this regard, research to elucidate the mechanisms underpinning EM in dairy cattle should not only focus on the pre-hatching period of development when most EM is believed to occur (Santos et al., 2004; Diskin and Morris, 2008) but also on the period of elongation when the process of CL maintenance occurs. Because immediately before and during the period of CL maintenance by IFNT, the conceptus is undergoing rapid growth and elongation (Hue et al., 2007; Spencer, 2013), the potential causes of EM may be different than during the period from fertilization to the beginning of elongation.

Another objective of this experiment was to evaluate follicular dynamics in cows that underwent EM because it has not been studied in detail. Interestingly, we did not observe substantial alterations of the follicular dynamics from the approximate time of the onset of luteal regression to ovulation in cows with EM. For cows that exhibited luteal regression and ovulated, the patterns of follicle growth and size (diameter) after the onset of luteal regression were not different for cows in the EM reproductive status group. It is important to note that there were cows that did not undergo complete luteal regression or ovulate 42 days after AI. Therefore, days to complete luteal regression and to ovulation were biased towards cows that had the shortest interovulatory intervals among cows with EM. The lack of difference in estradiol concentrations during the period of CL regression and periovulatory period also supported the notion that the presence and subsequent demise of an embryo did not cause direct or indirect (through endocrine changes) alterations to the steroidogenic capacity of follicles. Unfortunately, we were unable to determine if alterations to follicle development before the onset of luteolysis were responsible, at

least in part, for the delay in the onset of luteal regression in cows with EM because the exact timing of EM could not be determined.

Taken together, the results for luteal dynamics and follicle development in lactating dairy cows that suffered EM around the period of CL maintenance by IFNT suggest that ovarian function alterations were mostly due to delayed luteal regression and not because of major alterations of follicular dynamics. Thus, further research should focus on understanding the mechanism underpinning the onset of luteal regression after EM. In cyclic cows, or in the absence of an embryo during the expected period of CL maintenance by IFNT, the mechanism responsible for luteolysis depends upon a series of well-orchestrated hormone release and receptor expression patterns in the uterus and ovaries (McCracken et al., 1999; Niswender et al., 2000; Okuda et al., 2002). In pregnant cows, the presence of the embryo and its secretions (e.g., IFNT and other pregnancy-specific proteins) cause profound alterations to these patterns to prevent luteolysis and sustain pregnancy. As a result, the series and timing of endocrine events that lead to the reestablishment of the luteolytic mechanism in cows that suffer EM may be different than in cyclic cows.

CONCLUSIONS

Our current results provided evidence that the combination of ISG mRNA relative abundance and circulating PSPB (marker of a suite of PAG) concentrations after AI is a reasonable method to experimentally determine the presence or absence of an embryo around the period of CL maintenance by IFNT. Cows with evidence of EM based on ISG and PSPB exhibited a pattern of ISG expression that more closely resembled that of pregnant than cyclic

cows or cows with no evidence of embryo presence during this period of time. This study also highlights the substantial number of pregnancies that are present during the period of CL maintenance by IFNT that are subsequently lost.

Among cows with EM, only those with LEM had delayed luteal regression, reduced ovulation rate, and longer interovulatory intervals compared with cyclic cows and cows with no evidence of the presence of an embryo during the period of CL maintenance by IFNT. Thus, our results suggest that embryo development needs to continue beyond 22 days after AI to effectively prevent luteolysis and extend the luteal phase substantially. Cows in the EEM reproductive status group exhibited evidence of IFNT secretion but the amount and timing of IFNT might have been insufficient to effectively prevent luteolysis. Finally, ovarian function alterations in cows that underwent EM were mostly due to delayed luteal regression rather than alterations of follicle development after the onset of luteolysis.

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REFERENCES

Bartolome, J., A. Sozzi, J. McHale, P. Melendez, A. Arteche, F. Silvestre, D. Kelbert, K. Swift, L. Archbald, and W. Thatcher. 2005. Resynchronization of ovulation and timed insemination in lactating dairy cows, II: assigning protocols according to stages of the estrous cycle, or presence of ovarian cysts or anestrus. Theriogenology 63:1628-1642.

Breukelman, S. P., Z. Perenyi, M. A. Taverne, H. Jonker, G. C. van der Weijden, P. L. Vos, L. de Ruigh, S. J. Dieleman, J. F. Beckers, and O. Szenci. 2012. Characterisation of pregnancy losses after embryo transfer by measuring plasma progesterone and bovine pregnancy-associated glycoprotein-1 concentrations. Vet J 194:71-76.

Bruno, R., J. Moraes, J. Hernández-Rivera, K. Lager, P. Silva, A. Scanavez, L. Mendonça, R. Chebel, and T. Bilby. 2014. Effect of an Ovsynch56 protocol initiated at different intervals after insemination with or without a presynchronizing injection of gonadotropin-releasing hormone on fertility in lactating dairy cows. Journal of dairy science 97:185-194.

Cerri, R., I. Thompson, I. Kim, A. Ealy, P. Hansen, C. Staples, J. Li, J. Santos, and W. Thatcher. 2012. Effects of lactation and pregnancy on gene expression of endometrium of Holstein cows at day 17 of the estrous cycle or pregnancy. Journal of dairy science 95:5657-5675.

Chebel, R., J. Santos, R. Cerri, H. Rutigliano, and R. Bruno. 2006. Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. Journal of dairy science 89:4205-4219.

Diskin, M. and D. Morris. 2008. Embryonic and early foetal losses in cattle and other ruminants. Reproduction in Domestic Animals 43:260-267.

Farin, C., K. Imakawa, T. Hansen, J. McDonnell, C. Murphy, P. Farin, and R. Roberts. 1990. Expression of trophoblastic interferon genes in sheep and cattle. Biology of Reproduction 43:210-218.

Gábor, G., F. Tóth, L. Ozsvári, Z. Abonyi-Tóth, and R. Sasser. 2007. Early detection of pregnancy and embryonic loss in dairy cattle by ELISA tests. Reproduction in domestic animals 42:633-636.

Gifford, C. A., K. Racicot, D. S. Clark, K. J. Austin, T. R. Hansen, M. C. Lucy, C. J. Davies, and T. L. Ott. 2007. Regulation of interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant dairy cows. J Dairy Sci 90:274-280.

Giordano, J., J. Guenther, G. Lopes, and P. Fricke. 2012a. Changes in serum pregnancy-associated glycoprotein, pregnancy-specific protein B, and progesterone concentrations before and after induction of pregnancy loss in lactating dairy cows. Journal of dairy science 95:683-697.

Giordano, J., M. Stangaferro, R. Wijma, W. Chandler, and R. Watters. 2015. Reproductive performance of dairy cows managed with a program aimed at increasing insemination of cows in estrus based on increased physical activity and fertility of timed artificial inseminations. Journal of dairy science 98:2488-2501.

Giordano, J., M. Wiltbank, J. Guenther, R. Pawlisch, S. Bas, A. Cunha, and P. Fricke. 2012b. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. Journal of dairy science 95:639-653.

Green, J., C. Okamura, S. Poock, and M. Lucy. 2010. Measurement of interferon-tau (IFN-τ) stimulated gene expression in blood leukocytes for pregnancy diagnosis within 18–20d after insemination in dairy cattle. Animal reproduction science 121:24-33.

Green, J. A., T. E. Parks, M. P. Avalle, B. P. Telugu, A. L. McLain, A. J. Peterson, W. McMillan, N. Mathialagan, R. R. Hook, and S. Xie. 2005. The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. Theriogenology 63:1481-1503.

Grimard, B., S. Freret, A. Chevallier, A. Pinto, C. Ponsart, and P. Humblot. 2006. Genetic and environmental factors influencing first service conception rate and late embryonic/foetal mortality in low fertility dairy herds. Animal reproduction science 91:31-44.

Han, H., K. J. Austin, L. A. Rempel, and T. R. Hansen. 2006. Low blood ISG15 mRNA and progesterone levels are predictive of non-pregnant dairy cows. J Endocrinol 191:505-512.

Hansen, T. R., N. P. Smirnova, H. Van Campen, M. L. Shoemaker, A. A. Ptitsyn, and H. Bielefeldt-Ohmann. 2010. Maternal and Fetal Response to Fetal Persistent Infection with Bovine Viral Diarrhea Virus*. American Journal of Reproductive Immunology 64:295-306.

Helmer, S., P. Hansen, W. Thatcher, J. Johnson, and F. Bazer. 1989. Intrauterine infusion of highly enriched bovine trophoblast protein-1 complex exerts an antiluteolytic effect to extend corpus luteum lifespan in cyclic cattle. Journal of reproduction and fertility 87:89-101.

Heyman, Y., S. Camous, J. Fevre, W. Méziou, and J. Martal. 1984. Maintenance of the corpus luteum after uterine transfer of trophoblastic vesicles to cyclic cows and ewes. Journal of reproduction and fertility 70:533-540.

Hubbert, W. 1972. Committee on bovine reproductive nomenclature. Recommendations for standardizing bovine reproductive terms. Cornell Vet 62:216-237.

Hue, I., S. Degrelle, E. Campion, and J. Renard. 2007. Gene expression in elongating and gastrulating embryos from ruminants. Society of Reproduction and Fertility supplement 64:365.

Humblot, P. 2001. Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. Theriogenology 56:1417-1433.

Knickerbocker, J., W. Thatcher, F. Bazer, M. Drost, D. Barron, K. Fincher, and R. Roberts. 1986. Proteins secreted by day-16 to-18 bovine conceptuses extend corpus luteum function in cows. Journal of reproduction and fertility 77:381-391.

Liebig BE, Thomas GM, Mc Sweeney KD, Van Campen H, Bishop J, and Hansen TH. Fertility may depend on conceptus-derived signals in lactating dairy cows. J Dairy Sci 2015; 98(E-Supplement 1). Abstract LB1.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. methods 25:402-408.

Lopes, G., J. Giordano, A. Valenza, M. Herlihy, J. Guenther, M. Wiltbank, and P. Fricke. 2013. Effect of timing of initiation of resynchronization and presynchronization with gonadotropin-releasing hormone on fertility of resynchronized inseminations in lactating dairy cows. Journal of dairy science 96:3788-3798.

Mann, G., M. Fray, and G. Lamming. 2006. Effects of time of progesterone supplementation on embryo development and interferon-τ production in the cow. The Veterinary Journal 171:500-503.

Mann, G. and G. Lamming. 2001. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction 121:175-180.

Matsuyama, S., T. Kojima, S. Kato, and K. Kimura. 2012. Relationship between quantity of IFNT estimated by IFN-stimulated gene expression in peripheral blood mononuclear cells and bovine embryonic mortality after AI or ET. Reprod Biol Endocrinol 10:21.

McCracken, J. A., E. E. Custer, and J. C. Lamsa. 1999. Luteolysis: a neuroendocrine-mediated event. Physiological reviews 79:263-323.

McGlone, J. 2010. Guide for the care and use of agricultural animals in research and teaching.

Meyer, M., P. Hansen, W. Thatcher, M. Drost, L. Badinga, R. Roberts, J. Li, T. Ott, and F. Bazer. 1995. Extension of corpus luteum lifespan and reduction of uterine secretion of prostaglandin F 2α of cows in response to recombinant interferon-τ. Journal of dairy science 78:1921-1931.

Moore, D. A., M. W. Overton, R. C. Chebel, M. L. Truscott, and R. H. BonDurant. 2005. Evaluation of factors that affect embryonic loss in dairy cattle. Journal of the American Veterinary Medical Association 226:1112-1118.

Moreira, F., C. Orlandi, C. Risco, R. Mattos, F. Lopes, and W. Thatcher. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. Journal of Dairy Science 84:1646-1659.

Niswender, G. D., J. L. Juengel, P. J. Silva, M. K. Rollyson, and E. W. McIntush. 2000. Mechanisms controlling the function and life span of the corpus luteum. Physiological reviews 80:1-29.

Northey, D. and L. French. 1980. Effect of embryo removal and intrauterine infusion of embryonic homogenates on the lifespan of the bovine corpus luteum. Journal of animal science 50:298-302.

Okuda, K., Y. Miyamoto, and D. Skarzynski. 2002. Regulation of endometrial prostaglandin F 2α synthesis during luteolysis and early pregnancy in cattle. Domestic animal endocrinology 23:255-264.

Plante, C., D. Bousquet, P. Guay, and A. Goff. 1984. Embryonic tissue associated with the embryonic signal in early pregnancy. Theriogenology 21:253.

Pugliesi, G., B. T. Miagawa, Y. N. Paiva, M. R. França, L. A. Silva, and M. Binelli. 2014. Conceptus-induced changes in the gene expression of blood immune cells and the ultrasound-

accessed luteal function in beef cattle: how early can we detect pregnancy? Biology of reproduction 91:95.

Remnant, J., M. Green, J. Huxley, and C. Hudson. 2015. Variation in the interservice intervals of dairy cows in the United Kingdom. Journal of dairy science 98:889-897.

Ribeiro, E., A. Monteiro, R. Bisinotto, F. Lima, L. Greco, A. Ealy, W. Thatcher, and J. Santos. 2016a. Conceptus development and transcriptome at preimplantation stages in lactating dairy cows of distinct genetic groups and estrous cyclic statuses. Journal of Dairy Science.

Ribeiro, E. S., R. G. Bruno, A. M. Farias, J. A. Hernández-Rivera, G. C. Gomes, R. Surjus, L. F. Becker, A. Birt, T. L. Ott, and J. R. Branen. 2014. Low doses of bovine somatotropin enhance conceptus development and fertility in lactating dairy cows. Biology of reproduction 90:10.

Ribeiro, E. S., L. F. Greco, R. S. Bisinotto, F. S. Lima, W. W. Thatcher, and J. E. Santos. 2016b. Biology of Preimplantation Conceptus at the Onset of Elongation in Dairy Cows. Biology of reproduction:biolreprod. 115.134908.

Roberts, R. M., S. Xie, and N. Mathialagan. 1996. Maternal recognition of pregnancy. Biology of reproduction 54:294-302.

Robinson, R., M. Fray, D. Wathes, G. Lamming, and G. Mann. 2006. In vivo expression of interferon tau mRNA by the embryonic trophoblast and uterine concentrations of interferon tau protein during early pregnancy in the cow. Molecular reproduction and development 73:470-474.

Santos, J., W. Thatcher, R. Chebel, R. Cerri, and K. Galvao. 2004. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. Animal Reproduction Science 82:513-535.

Shirasuna, K., H. Matsumoto, E. Kobayashi, A. Nitta, S. Haneda, M. Matsui, C. Kawashima, K. Kida, T. Shimizu, and A. Miyamoto. 2012. Upregulation of interferon-stimulated genes and interleukin-10 in peripheral blood immune cells during early pregnancy in dairy cows. Journal of Reproduction and Development 58:84-90.

Smirnova, N. P., B. T. Webb, H. Bielefeldt-Ohmann, H. Van Campen, A. Q. Antoniazzi, S. E. Morarie, and T. R. Hansen. 2012. Development of fetal and placental innate immune responses during establishment of persistent infection with bovine viral diarrhea virus. Virus research 167:329-336.

Spencer, T. E. 2013. Early pregnancy: Concepts, challenges, and potential solutions. Animal frontiers 3:48-55.

Spencer, T. E., G. A. Johnson, F. W. Bazer, R. C. Burghardt, and M. Palmarini. 2006. Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. Reproduction, Fertility and Development 19:65-78.

Stevenson, J., J. Dalton, T. Ott, K. Racicot, and R. Chebel. 2007. Correlation between reproductive status and steady-state messenger ribonucleic acid levels of the resistance gene, MX2, in peripheral blood leukocytes of dairy heifers. Journal of animal science 85:2163-2172.

Weiner, C., N. Smirnova, B. Webb, H. Van Campen, and T. Hansen. 2012. Interferon stimulated genes, CXCR4 and immune cell responses in peripheral blood mononuclear cells infected with bovine viral diarrhea virus. Research in veterinary science 93:1081-1088.

Supplementary table 2.1. Individual *ISG15* and *MX2* fold changes (Days 18, 20 and 22 after AI) and pregnancy-specific protein B (PSPB; Days 24 and 26 after AI) concentrations for cows in the Embryo Mortality reproductive status group.

	Day 18		Day 20		Day 22		Day 24	Day 26	Outcome	
Cow ID	*ISG15*	*MX2*	*ISG15*	*MX2*	*ISG15*	*MX2*	PSPB (ng/mL)	PSPB (ng/mL)	ISG +	PSPB +
8849	1.0	1.8	1.9	1.7	2.3	3.9	0.6	1.2	-	+
8964	4.0	4.3	2.1	2.9	4.2	3.1	0.7	1.6	+	+
9168	6.8	1.7	45.6	14.6	12.1	7.2	0.9	1.2	+	+
9441	1.5	3.7	6.5	4.0	8.3	5.8	0.6	0.7	+	+
9538	3.0	2.3	15.2	7.2	15.3	7.7	1.1	1.9	+	+
9547	0.8	0.8	0.6	1.1	0.9	1.4	0.5	0.8	-	+
9578*	0.8	0.7	2.7	1.3	1.2	0.9	< 0.3	0.4	-	+
8066	2.7	1.5	5.0	4.3	1.5	2.8	< 0.3	< 0.3	+	-
8442	4.9	12.7	2.2	3.9	1.5	2.4	< 0.3	< 0.3	+	-
8699	5.6	4.6	6.1	5.3	2.9	3.4	< 0.3	< 0.3	+	-
9197	0.5	1.8	0.3	5.3	0.5	5.3	< 0.3	< 0.3	+	-
9321	5.5	0.8	14.5	1.4	5.1	1.0	< 0.3	< 0.3	+	-
9518	26.7	30.7	7.0	7.0	1.0	1.4	< 0.3	< 0.3	+	-
9611	4.3	7.1	0.6	11.7	3.8	2.9	< 0.3	< 0.3	+	-

*reached 0.7 ng/mL of PSPB in plasma on Day 32.

CHAPTER III

RESYNCHRONIZATION OF OVULATION PROTOCOLS FOR DAIRY COWS INCLUDING OR NOT INCLUDING GNRH TO INDUCE A NEW FOLLICULAR WAVE: EFFECTS ON RE-INSEMINATION PATTERN, OVARIAN RESPONSES, AND PREGNANCY OUTCOMES

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ABSTRACT

Our objectives were to evaluate the pattern of re-insemination, ovarian responses, and pregnancy per AI (P/AI) of cows submitted to different resynchronization of ovulation protocols. The base protocol started at 25 ± 3 d after AI and was as follows: GnRH, 7 and 8 d later PGF2α, GnRH 32 h after second PGF2α, and fixed timed AI (TAI) 16 to 18 h after GnRH. At 18 ± 3 d after AI cows were randomly assigned to the G25 (n = 1,100) or NoG25 (n = 1,098) treatments. The protocol for G25 and NoG25 was the same, except that cows in NoG25 did not receive GnRH 25 ± 3 d after AI. At nonpregnancy diagnosis (NPD), 32 ± 3 d after AI, cows from G25 and NoG25 with a corpus luteum (CL) ≥ 15 mm in diameter and a follicle ≥ 10 mm completed the protocol (G25 CL = 272, NoG25 CL = 194), whereas cows from both treatments that did not meet these criteria received a modified Ovsynch protocol with P4 supplementation [controlled internal drug release insert (CIDR) plus GnRH, CIDR removal and PGF2α 7 and 8 d later, GnRH 32 h after second PGF2α, and TAI 16 to 18 h after GnRH (G25 NoCL = 53, NoG25 NoCL = 78)]. Serum concentrations of progesterone (P4) were determined and ovarian ultrasonography was performed thrice weekly from 18 ± 3 d after AI until one day after TAI (G25 = 46, NoG25 = 44 cows). A greater percentage of NoG25 cows were re-inseminated at

detected estrus (NoG25 = 53.5%, G25 = 44.6%), whereas more cows had a CL at NPD in G25 than NoG25 (83.7 and 71.3%). At 32 d after AI, P/AI was similar for G25 and NoG25 for inseminations at detected estrus (38.4 and 42.9%), TAI services for cows with no CL (40.4 and 36.7%), and for all services combined (39.6 and 39.0%). However, P/AI were greater for cows with a CL in G25 than NoG25 (40.6 and 32.8%) that received TAI. More cows ovulated spontaneously or in response to GnRH for the G25 than the NoG25 treatment (70 and 36%) but a similar proportion had an active follicle at NPD (G25 = 91% and NoG25 = 96%). Largest follicle diameter at NPD (G25 = 15.0 ± 0.4 mm, NoG25 = 16.5 ± 0.6 mm) and days since it reached ≥ 10 mm (G25 = 4.0 ± 0.3 d, NoG25 = 5.8 ± 0.6 d) was greater for the NoG25 than G25 treatment. For cows with a CL at NPD, CL regression after NPD, ovulation after TAI, and ovulatory follicle diameter did not differ. In conclusion, removing the first GnRH of a modified Resynch-25 protocol for cows with a CL at NPD and a modified Ovsynch protocol with P4 supplementation for cows without a CL at NPD resulted in a greater percentage of cows re-inseminated at detected estrus and a similar proportion of cows pregnant in spite of reduced P/AI for cows with a CL at NPD.

Keyword: resynchronization, estrus detection, GnRH, dairy cow

INTRODUCTION

An ideal strategy for submitting cows for re-insemination minimizes the interbreeding interval and maximizes pregnancy per AI (P/AI). Many dairy farms reduce the interbreeding interval by combining re-insemination of cows after a detected estrus and submission to resynchronization of ovulation for timed AI [TAI; (Caraviello et al., 2006; Ferguson and Skidmore, 2013; Scott, 2016)]. Re-insemination of cows at estrus with resulting P/AI similar to

or better than that observed after re-insemination at fixed time AI benefits herd profitability by reducing the overall interbreeding interval and reproductive program cost (Giordano et al., 2012b; Galvão et al., 2013; Giordano et al., 2013). On the other hand, incorporating a resynchronization of ovulation protocol for TAI is essential because it ensures timely re-insemination of nonpregnant cows not detected in estrus. Therefore, Ovsynch-type protocols are usually initiated at the time of or seven days before nonpregnancy diagnosis (NPD) to ensure that nonpregnant cows receive TAI as soon as possible (Chebel et al., 2003; Fricke et al., 2003; Lopes et al., 2013).

For farms that enroll cohorts of cows in a resynchronization of ovulation protocol on a weekly basis, an effective strategy to minimize the interbreeding interval of TAI services is to initiate the Ovsynch protocol (GnRH, 7 d later PGF2α, 56 h later GnRH, and 16 to 20 h later TAI) 25 ± 3 d after AI (hereafter referred to as Resynch-25), which results in an interbreeding interval of 35 ± 3 d. A caveat of this protocol is that the GnRH treatment 25 ± 3 d after AI may reduce estrus expression because many cows are expected to receive the treatment when a follicle capable of ovulating in response to a GnRH-induced LH surge is present on the ovaries. In cattle, a GnRH-induced LH surge causes an immediate reduction in estradiol production by the dominant follicle (Berndtson et al., 1995; Komar et al., 2001; Jo and Fortune, 2003), which can prevent the estradiol surge responsible for estrous behavior. Indeed, previous research has shown that fewer cows are detected in estrus when GnRH is given to cows 17 to 32 d after AI (Chebel et al., 2003; Mendonça et al., 2012; Bruno et al., 2014). Another inconvenience associated with the Resynch-25 protocol is that because the pregnancy status of cows is unknown 25 ± 3 d after AI, a substantial proportion of the cows that receive the first GnRH treatment are pregnant. These unnecessary treatments increase the cost and burden (i.e., labor and cow

disruption) of the reproductive management program. Thus, removing the first GnRH treatment of the protocol may be an alternative strategy to reduce the interbreeding interval without disrupting re-insemination at estrus and eliminating the treatment of pregnant cows.

The first GnRH of the Resynch-25 protocol is meant to induce the emergence of a new follicular wave that will give rise to the ovulatory follicle and the formation of a new corpus luteum (CL) after ovulation (Pursley et al., 1995). Therefore, removing the first GnRH of the Resynch-25 protocol may reduce the synchrony of ovulation, fail to promote a proper endocrine environment for follicle growth, and result in poorer oocyte quality because of an extended period of dominance of the ovulatory follicle. Indeed, P/AI of cows that ovulate to the first GnRH treatment of Ovsynch-like protocols is greater than that of cows that fail to ovulate (Chebel et al., 2003; Galvão et al., 2007; Rutigliano et al., 2008; Bisinotto et al., 2010; Keskin et al., 2010; Giordano et al., 2012d). Nonetheless, the detrimental effects of lack of ovulation may be less relevant when initiating Resynch 25 ± 3 d after AI than when initiating the protocol at other intervals after AI (e.g., 32 ± 3 or 39 ± 3 d) or when a group of cows is initiated in the protocol at a wider range of days after AI. This is because at 32 ± 3 d after AI, when cows would receive the PGF2α treatment of the Resynch-25 protocol, 60 to 85% of the nonpregnant cows present a functional CL (Giordano et al., 2012d; Bruno et al., 2014; Giordano et al., 2015; Wijma et al., 2015) and almost all cows present an active follicle with potential to continue growing after a PGF2α treatment (Wijma et al., 2015). Moreover, the ovulatory response to the first GnRH of the Resynch-25 protocol rarely exceeds ~40 to 60% (Giordano et al., 2012c; Lopes et al., 2013; Bruno et al., 2014), resulting in not all cows properly synchronized 7 d later.

Another advantage of management strategies aimed at reducing the interbreeding interval of TAI services is that NPD is usually conducted through transrectal ultrasonography (Chebel et

al., 2003; Fricke et al., 2003; Giordano et al., 2015). This method of pregnancy testing allows the determination of the ovarian status of cows with minimal additional effort. Anticipating that the use of the Resynch-25 protocol without the GnRH treatment 25 ± 3 d after AI could result in fewer cows at the appropriate stage of the estrous cycle at NPD; using treatments tailored to the ovarian status of cows could help maximize P/AI to TAI services (Giordano et al., 2016). For example, completing the Resynch-25 protocol only in cows that present a CL and a putative ovulatory follicle at NPD could result in reasonable P/AI. Conversely, cows without a CL and/or a putative ovulatory follicle may benefit from re-enrollment in a synchronization of ovulation treatment including progesterone (P4) supplementation (Bisinotto et al., 2015; Giordano et al., 2016) through improved synchrony of ovulation and a better endocrine environment before TAI.

Thus, our objectives were to evaluate the effect of removing the first GnRH of the Resynch-25 protocol on the pattern of re-insemination, ovarian responses before and after the protocol, and P/AI. We hypothesized that removing the GnRH treatment 25 ± 3 d after AI would result in re-insemination of more cows upon detection of estrus. A secondary hypothesis was that the expected reduction in P/AI for TAI services in cows that did not receive the GnRH treatment, would not reduce the overall proportion of pregnant cows. We expected that cows pregnant after insemination at detected estrus would compensate for the reduced fertility to TAI services.

MATERIALS AND METHODS

This experiment was conducted from March 2015 to December of 2016 at the Dairy Unit of the Cornell University Ruminant Center (Harford, NY). All procedures were approved by the Animal Care and Use Committee of Cornell University.

Cows were housed in free stall barns and were fed a total mixed ration once a day with ad libitum access to feed and water. The diet was formulated to meet or exceed nutrient requirements for lactating dairy cows producing 45 kg of milk per day (Cornell Net Carbohydrate and Protein System). Cows were milked thrice daily at approximately 8 h intervals and received recombinant bovine somatotropin (500 mg of Sometribove zinc; Posilac, Elanco Animal Health, Indianapolis, IN) at 14 d intervals beginning at 60 ± 3 DIM until dry off.

Cows received the first service postpartum at 64 ± 3 DIM after synchronization of ovulation with the Presynch-Ovsynch protocol [PGF2α, 14 d later PGF2α, 12 d later GnRH, 7 d later PGF2α, 56 h later GnRH, and 16 to 18 h later fixed time AI (Moreira et al., 2001)]. Ten percent of the cows did not complete the synchronization protocol and received the first service at detected estrus after the second PGF2α treatment of Presynch because of management decisions from the farm manager.

Every Monday, the cohort of cows at 18 ± 3 d after AI were blocked by parity (primiparous vs. multiparous) and randomly assigned to receive (G25, n = 1,100) or not receive (NoG25, n = 1,098) GnRH (Cystorelin, 100 µg, Merial LLC, Duluth, GA) treatment 25 ± 3 d after AI (Figure 3.1). Cows could have been enrolled in either one of the treatments more than once. At 32 ± 3 d after AI, NPD was performed by transrectal ultrasonography (Ibex Pro, Ibex, Loveland, CO) in cows from both treatments not previously re-inseminated at detected estrus. A cow was considered pregnant if a viable embryo with a heartbeat or uterine fluid and a CL ≥ 15 mm were observed. Pregnant cows received no further treatment, whereas nonpregnant cows from both treatments (G25 and NoG25) were classified based on the ovarian structures present. Cows from the G25 and NoG25 treatments with at least one CL ≥ 15 mm and a follicle ≥ 10 mm in diameter received a PGF2α (Estrumate, 500 µg, Merck Animal Health, Summit, NJ, USA)

treatment immediately after NPD, a second PGF2α treatment 24 h later, GnRH 32 h after the second PGF2α treatment, and TAI 16 to 18 h later (Figure 3.1). Cows from the G25 and NoG25 treatments that did not meet these criteria received a modified Ovsynch protocol with two PGF2α treatments and P4 supplementation through an intravaginal P4 releasing device (Eazi-Breed CIDR, Zoetis Animal Health, Florham Park, NJ) from the time of NPD to the first PGF2α of the protocol (GnRH plus CIDR insertion, 7 d later PGF2α and CIDR removal, 24 h later PGF2α, 32 h later GnRH, and 16 to 18 h later TAI; Figure 3.1).

For the G25 treatment, 272 cows with a CL and 53 cows without a CL received TAI, whereas for the NoG25 treatment 194 cows with a CL and 78 cows without a CL received TAI.

All cows detected in estrus after AI (before or after enrollment) were immediately inseminated. Detection of estrus was conducted through visual observation twice a day for ~30 to 45 min per session by the technician in charge of implementing the farm reproductive management program (i.e., artificial insemination, hormonal treatments, and assisting research personnel during pregnancy testing). Farm personnel in charge of moving cows to and from the parlor and milking also recorded estrus events.

Reconfirmation of pregnancy was performed at 63 ± 3 d after AI by transrectal palpation of the uterine contents. Pregnancy loss was defined as a negative pregnancy outcome at re-confirmation of pregnancy for a cow pregnant at the initial pregnancy examination or when a cow received insemination at detected estrus between the two pregnancy examinations.

Evaluation of Ovarian Responses

In a sub-group of cows (n = 362) from each treatment (G25 = 166, NoG25 = 196), ovarian responses were monitored by transrectal ultrasonography following a Monday,

Wednesday, and Friday schedule (enrollment and NPD were performed on Mondays) from 18 ± 3 d after AI until cows were either diagnosed pregnant or re-inseminated. Two hundred and seventy two cows were either detected in estrus and inseminated before NPD or were pregnant, thereby the final number of cows with data available from enrolment until TAI was 46 and 44 for the G25 and NoG25 treatment, respectively.

At each examination, the location and size (diameter) of follicles ≥ 8 mm and any corpora lutea present was recorded on an individual ovarian map. All measurements were performed using the ultrasound machine internal digital calipers. Follicle and CL diameter was the average of the two longest perpendicular measurements. Ovulation was defined as the disappearance of at least one putative ovulatory follicle ≥ 10 mm between two consecutive examinations. The day of ovulation was defined as the day previous to the examination when follicle disappearance was detected. Ovulations detected up to 48 h after the GnRH treatment 25 ± 3 d after AI were considered induced by GnRH, whereas ovulations before GnRH treatment or more than 48 h after GnRH treatment were considered spontaneous ovulations. Follicles were considered active (i.e., growing or static phase of development) when their diameter was ≥ 10 mm and either continued to grow or did not present two consecutive reductions in size in three consecutive examinations.

Blood Sample Collection

Blood samples were collected at the time of the transrectal ultrasonography examination from the subgroup of cows in which ovarian responses were monitored (G25 = 166, NoG25 = 196). Blood samples were collected using 8-mL serum separator tubes (BD Vacutainer, Franklin Lakes, NJ) via puncture of the coccygeal vein or artery and were used to determine circulating

concentrations of P4. Samples were centrifuged at 1,700 x g for 20 minutes at room temperature within 2 h of collection. Serum was harvested and transferred into different storage vials and stored at -20°C until assays were performed.

Determination of Progesterone Concentrations

Progesterone concentrations in serum were determined in duplicate using a commercial solid-phase, no-extraction radioimmunoassay (ImmuChem Coated Tube, MP Biomedicals, Costa Mesa, CA). To assess precision of the assay, control samples with high (4.5 ng/mL) and low (0.3 ng/mL) concentrations of P4 were included at the beginning and end of each assay (n = 7 assays). Average detection limit for the P4 assay was 0.1 ng/mL. Average intra-assay CV for the high-concentration sample was 13%, whereas the inter-assay CV was 16%. For the low-concentration sample the average intra-assay CV was 22%, whereas the inter-assay CV was 26%.

Circulating concentrations of P4 were used to determine the presence or absence of a functional CL and luteal regression. The presence of a functional CL was defined as circulating concentrations of P4 $\geq$ 0.5 ng/mL at time points of interest, whereas luteal regression was defined as drop in circulating concentrations of P4 to < 0.5 ng/mL.

Statistical Analysis

The experiment was conducted as a complete randomized design using parity (primiparous vs. multiparous) as blocking factor, and number of AI services as stratification factor. Because the experimental unit was the AI service after enrollment, cows were randomized each time they reached 18 ± 3 d after a previous AI.

A sample size calculation was performed using the sample size calculation option of WinPepi version 11.51 (Abramson, 2011). Based on an expected difference between treatments in the proportion of cows detected in estrus of 10 percentage points for cows that received GnRH treatment at the same range of days after AI (Bruno et al., 2014), a total of 325 cows per treatment was needed for a one-tailed test with probability of type I error rate of 5% and probability of type II error rate of 20%. Moreover, because P/AI for cows with a CL at NPD that did not receive GnRH was a secondary outcome of interest for this experiment, we planned on enrolling more cows to have sufficient experimental units (i.e., AI services) to detect a difference in P/AI ranging from 11 to 12 percentage points when P/AI for TAI services in cows with a CL in the G25 treatment was 35%. A total of 176 to 212 cows per treatment were needed for a one-tailed test with probability of type I error rate of 5% and probability of type II error rate of 20%. We did not expect to detect a difference in P/AI for cows inseminated after a detected estrus or cows without a CL at NPD. A one-tailed test was used for the analysis of proportion of cows detected in estrus and P/AI for cows with CL at NPD because in both cases the hypothesis was that the difference between treatments would be in one direction. Specifically, we anticipated observing a greater proportion of cows detected in estrus in the NoG25 treatment based on experiments that reported a reduction in estrus expression in cows treated with GnRH (Bruno et al., 2014). For P/AI in cows with a CL, we anticipated observing greater P/AI for the G25 treatment because cows that ovulated in response to the GnRH treatment to induce a follicular wave in Ovsynch-type protocols had greater P/AI in previous experiments (Chebel et al., 2003; Bisinotto et al., 2010; Keskin et al., 2010).

Binary outcomes (P/AI, proportion of cows detected in estrus, proportion of cows with a CL at NPD, pregnancy loss from 32 ± 3 to 63 ± 3 d after AI, ovulation, presence of an active

follicle at NPD, and proportion of cows with P4 ≥ 0.5 ng/mL at NPD) were analyzed using logistic regression with the GLIMMIX procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Treatment and parity (primiparous vs. multiparous) were included as fixed effects, and cow was included as random effect when applicable (proportion of cows detected in estrus, proportion of cows with a CL at NPD). Days in milk at enrollment and season at the time of insemination [warm (June, July, and August) vs. cold (September to May)] were offered to the initial models. Both DIM and season were removed from all final models because $P > 0.10$. Luteal regression and presence of an active follicle at the time of GnRH treatment 25 ± 3 d after AI were analyzed with the Fisher exact test using the FREQ procedure of SAS because in one of the groups 100% of the cows presented luteal regression or an active follicle.

Data for circulating concentrations of P4, follicle size, days to ovulation, and days after the largest active follicle present 25 ± 3 and 32 ± 3 d after AI reached 10 mm were analyzed by ANOVA with the MIXED procedure of SAS including treatment and parity as fixed effects. Normality and homoscedasticity of residuals were assessed using graphical methods generated with the residual option of the MIXED procedure of SAS. Because progesterone concentration data were not normally distributed, square root transformation of the data was used. Days to ovulation and days after the largest active follicle present 25 ± 3 and 32 ± 3 d after AI reached 10 mm did not follow a normal distribution and could not be corrected through data transformation. Therefore, data were analyzed using the Kruskal-Wallis test with the NPAR1WAY procedure of SAS.

Cox's proportional hazards regression run with the PHREG procedure of SAS was used to determine the hazard of ovulation and re-insemination after AI. Treatment and parity were included as fixed effects, and cow as a random effect (for hazard of re-insemination only).

Kaplan-Meier survival curves were generated to illustrate the hazard of re-insemination in estrus after AI using the Survival Analysis option of MedCalc (MedCalc Software bvba, Ostend, Belgium).

All results are presented as arithmetic means ± SE generated with the MEANS procedure of SAS. All explanatory variables were considered significant if $P \leq 0.05$, whereas P-values > 0.05 and ≤ 0.10 were considered a tendency.

RESULTS

Re-insemination Dynamics and Pregnancy per Artificial Insemination

Forty four percent of the cows that were enrolled 18 ± 3 d after AI were considered pregnant based on transrectal ultrasonography 32 ± 3 d after AI. Pregnancy per AI for these cows was similar ($P = 0.81$) for the G25 (44.4%; 483/1,088) and the NoG25 (43.9%; 479/1,092) treatment.

A greater proportion of cows were inseminated ($P < 0.01$) after a detected estrus in the NoG25 than in the G25 treatment (Table 3.1). Parity also had an effect on the proportion of cows that received AI after estrus detection [primiparous = 55.3% (140/253) and multiparous = 47.3% (435/919); $P = 0.03$]. The hazard of insemination after estrus detection was greater ($P = 0.04$) for cows in the NoG25 treatment [hazard ratio (HR) = 1.20, 95% CI: 1.01 to 1.43; Figure 3.2]. Median time to re-insemination at detected estrus for the NoG25 treatment was 31 d (95% CI: 27 to 35), whereas it could not be calculated for the G25 treatment because less than 50% of the cows were re-inseminated at detected estrus. Further, mean days to detection of estrus were 23.2 ± 0.3 and 22.5 ± 0.3 for the NoG25 and G25 treatment, respectively. Primiparous cows had greater ($P = 0.01$) hazard of insemination after estrus detection than multiparous cows (HR =

1.35, 95% CI: 1.07 to 1.71). Mean days to estrus detection were 22.1 ± 0.4 d primiparous cows and 23.1 ± 0.2 d for multiparous cows.

In spite of the difference in the proportion of cows detected in estrus and hazard ratio for detection of estrus before NPD, the hazard of re-insemination for all services combined (at detected estrus and TAI) was similar ($P = 0.80$) for both treatments (HR = 1.02, 95% CI: 0.90 to 1.14). Median days to re-insemination were 34 and 31 d for the G25 and NoG25 treatment, respectively, whereas mean days to re-insemination were 30.3 ± 0.3 and 29.9 ± 0.3 d for the G25 and NoG25 treatment, respectively.

Among cows diagnosed nonpregnant 32 ± 3 d after AI, a greater ($P < 0.001$) proportion of cows in the NoG25 treatment did not have a CL ≥ 15 mm at NPD (Table 3.1). Parity did not affect ($P = 0.89$) the proportion of cows without a CL ≥ 15 mm at NPD.

Pregnancy per AI for all AI services combined (after detected estrus and TAI) did not differ ($P = 0.80$) between treatments (Table 3.2) but was greater ($P < 0.01$) for primiparous (46.8%; 117/250) than multiparous cows (37.2%; 332/893). Further, P/AI did not differ for the G25 and NoG25 treatments for cows that received AI after a detected estrus ($P = 0.30$) or for cows without a CL ≥ 15 mm at NPD that received TAI ($P = 0.82$; Table 3.2). Pregnancy per AI for cows with a CL ≥ 15 mm at NPD that received TAI was greater ($P = 0.05$) for the G25 than the NoG25 treatment (Table 3.2).

Five cows from the G25 treatment and five cows from the NoG25 treatment exited the herd before pregnancy reconfirmation. Pregnancy per AI 63 ± 3 d after AI was not different ($P = 0.76$) between treatments (G25 = 36.2% and NoG25 = 35.6%) and was greater ($P < 0.01$) for primiparous (43.0%, 107/249) than multiparous cows (34.1%, 301/583). Pregnancy loss from the initial pregnancy evaluation at 32 ± 3 after AI until pregnancy reconfirmation 63 ± 3 d after AI

for all inseminations was similar ($P = 0.94$) for both treatments (Table 3.2). Further, pregnancy loss was not different between the G25 and the NoG25 treatments for cows inseminated at detected estrus (G25 = 7% and NoG25 = 6%; $P = 0.77$), cows that had a CL ≥ 15 mm at NPD and received TAI (G25 = 9% and NoG25 = 8%; $P = 0.94$), and cows that did not have a CL ≥ 15 mm at NPD and received TAI (G25 = 0% and NoG25 = 11%; $P = 0.97$).

Ovarian Responses from Enrollment until Nonpregnancy Diagnosis

Out of 362 cows with data for ovarian responses, 42% (n = 153) were pregnant and 58% (n = 209) were nonpregnant 32 ± 3 d after AI. Among nonpregnant cows, 57% (n = 119) were inseminated after detection of estrus before NPD and 43% (n = 90; G25: n = 46 and NoG25: n = 44) were diagnosed nonpregnant 32 ± 3 d after AI. All cows diagnosed nonpregnant received TAI. Blood samples were not available for two cows (one from the G25 and one from the NoG25 treatment) but ovarian data collected by transrectal ultrasonography was included in the analysis.

The proportion of cows not re-inseminated at detected estrus that ovulated spontaneously from 18 ± 3 to 32 ± 3 d after AI and was not affected by treatment ($P = 0.20$; Table 3.3) or parity ($P = 0.38$). Including spontaneous and induced ovulations, a greater proportion ($P < 0.01$) of cows ovulated in the G25 treatment (72%) than the NoG25 treatment (36%) from 18 ± 3 to 32 ± 3 d after AI. For cows in the G25 treatment, 50% ovulated in response to the GnRH treatment given 25 ± 3 d after AI (Table 3.3). Parity did not affect ($P = 0.47$) the proportion of cows that ovulated. Mean days from the previous AI to ovulation were greater ($P < 0.01$) for cows in the G25 (25.9 ± 0.4 d; range: 21 to 30 d) than the NoG25 treatment (23.7 ± 0.7 d; range: 19 to 29 d; Table 3.3). From the time of enrolment until NPD, the hazard of ovulation was greater ($P =$

0.04) for cows in the G25 treatment (HR = 1.89, 95% CI: 1.05 to 3.5; Figure 3.3). Median days to ovulation for the G25 treatment were 26 d (95% CI: 26 to 30). Median days to ovulation could not be calculated for the NoG25 treatment because less than 50% of the cows ovulated.

For both treatments combined, 90% (81/90) of the cows had an active follicle 25 ± 3 d after AI. For cows in the G25 treatment only, the proportion of cows with an active follicle on the day of GnRH 25 ± 3 d after AI was similar ($P = 0.99$) for cows that did (100%) or did not (96%) ovulate. Conversely, diameter of the largest active follicle was greater ($P < 0.001$) for cows that ovulated (17.8 ± 0.9 mm, range = 10 to 29 mm) than for cows that failed to ovulate in response to GnRH (13.7 ± 0.7 mm, range = 10 to 20 mm). The largest active follicle present 25 ± 3 d after AI reached a size of ≥ 10 mm earlier ($P = 0.02$) in cows that ovulated (5.6 ± 0.4 d before GnRH) than in cows that did not ovulate (3.8 ± 0.6 d before GnRH). Further, the proportion of cows with P4 ≥ 1 ng/mL at the time of the GnRH treatment did not differ ($P = 0.23$) for the group that ovulated or did not ovulate after GnRH [ovulated = 52% (12/23) and not ovulated = 70% (16/23)] but, cows that ovulated tended ($P = 0.07$) to have lesser concentrations of P4 (2.1 ± 0.6 ng/mL) than cows that did not ovulate (3.6 ± 0.6 ng/mL).

Treatment did not ($P = 0.67$) affect the proportion of cows with at least one active follicle at NPD (Table 3.3). Parity, however, tended ($P = 0.07$) to affect the proportion of cows with at least one active follicle at NPD [primiparous = 80% (12/15) and multiparous = 95% (71/75)]. Size of the largest active follicle at NPD was greater ($P = 0.01$) for the NoG25 than for the G25 treatment (Table 3.3) and was not affected ($P = 0.11$) by parity. Furthermore, mean days since the largest active follicle present at NPD reached a size of ≥ 10 mm, was greater ($P < 0.01$) for the NoG25 than for the G25 treatment (Table 3.3). A similar proportion of cows in both

treatments (P = 0.38; Table 3.3) had P4 ≥ 0.5 ng/mL at NPD. Parity did not affect (P = 0.59) the proportion of cows with P4 ≥ 0.5 ng/mL at NPD.

Among cows with a CL ≥ 15 mm at NPD, those in the NoG25 treatment had greater (P = 0.03) concentrations of P4 at NPD than cows in the G25 treatment (Table 3.4). The proportion of cows with luteal regression from the time of NPD to the GnRH treatment before TAI was similar for both treatments (P = 0.97; Table 3.4). Ninety five percent and 100% of the cows had complete luteal regression in the G25 and NoG25 treatment, respectively. Likewise, mean size of the ovulatory follicle one day before TAI (P = 0.15) and the proportion of cows that ovulated after TAI (P = 0.35) were similar for both treatments (Table 3.4). For cows without a CL at NPD, treatment did not affect concentrations of P4 at NPD (P = 0.28), luteal regression before TAI (P = 0.99), size of the putative ovulatory follicle (P = 0.68), or ovulation after TAI (P = 0.97; Table 3.4). Parity did not affect circulating concentrations of P4 at NPD (P > 0.10), size of the ovulatory follicle (P > 0.10), or proportion of cows that ovulated after TAI (P > 0.10) in cows with or without a CL at NPD.

Table 3.1. Proportion of cows inseminated at detected estrus before nonpregnancy diagnosis and proportion of cows with or without a corpus luteum ≥ 15 mm at nonpregnancy for the G25 and NoG25 treatments.

	Treatment [1]		*P*-value
	G25 % (n/n)	NoG25 % (n/n)	
Estrus detection and AI before NPD[2]	44.6 (262/587)	53.5 (313/585)	<0.01
CL[3] ≥ 15 mm at NPD	83.7 (272/325)	71.3 (194/272)	<0.001
No CL ≥ 15 mm at NPD	16.3 (53/325)	28.6 (78/272)	<0.001

[1]Treatment = 18 ± 3 d after AI cows were randomly assigned to receive (G25) or not (NoG25) GnRH 25 ± 3 d after AI. At 32 ± 3 d after AI, nonpregnant cows from both treatments with at least one CL ≥ 15 mm received PGF2α-24 h-PGF2α-32 h-GnRH-16 to 18 h-TAI. Cows without a CL received a modified Ovsynch protocol with two PGF2α treatments and progesterone supplementation.
[2]NPD = nonpregnancy diagnosis.
[3]CL = corpus luteum.

Table 3.2. Pregnancy per AI and pregnancy loss for cows that received the G25 and NoG25 treatment.

Item	Treatment[1]		*P*-value
	G25 % (n/n)	NoG25 % (n/n)	
Overall P/AI[2] 32 ± 3 d after AI for all inseminations	39.6 (226/571)	39.0 (223/572)	0.80
Overall P/AI 63 ± 3 d after AI for all inseminations[3]	36.2 (205/566)	35.6 (202/567)	0.76
Overall pregnancy loss for all inseminations[4]	7.2 (16/223)	7.3 (16/218)	0.94
P/AI for inseminations at detected estrus	38.4 (99/258)	42.9 (132/308)	0.30
P/AI for cows with CL[5] ≥ 15 mm at NPD[6]	40.6 (106/261)	32.8 (62/189)	0.05
P/AI for cows without a CL ≥ 15 mm at NPD	40.4 (21/52)	36.7 (29/75)	0.82

[1]Treatment = 18 ± 3 d after AI cows were randomly assigned to receive (G25 treatment) or not (NoG25 treatment) GnRH 25 ± 3 d after AI. At 32 ± 3 d after AI, nonpregnant cows from both treatments with at least one CL ≥ 15 mm received PGF2α-24 h-PGF2α-32 h-GnRH-16 to 18 h-TAI. Cows without a CL received a modified Ovsynch protocol with two PGF2α treatments and progesterone supplementation.

[2]P/AI = pregnancy per AI

[3]Five cows from the G25 treatment, and five cows from the NoG25 treatment exited the herd before reconfirmation of pregnancy.

[4]Three pregnant cows from the G25 treatment, and five cows from the NoG25 treatment exited the herd before reconfirmation of pregnancy. Pregnancy loss from 32 ± 3 d after AI to the day of herd exited was not observed for these cows.

[5]CL = corpus luteum

[6]NPD = nonpregnancy diagnosis

Table 3.3. Ovarian responses and response to resynchronization protocols from enrollment up to the time of nonpregnancy diagnosis for cows in the G25 and NoG25 treatment.

Item	Treatment[1]		*P*-value
	G25 (n = 46)	NoG25 (n = 44)	
Spontaneous ovulation before NPD[2] (%)	22	36	0.20
Ovulation after GnRH[3] 25 ± 3 d after AI (%)	50	-	-
Mean days to ovulation (d)	25.9 ± 0.4	23.7 ± 0.7	<0.01
Active follicle at NPD (%)	91	96	0.67
Active follicle size (mm) at NPD	15.0 ± 0.4	16.5 ± 0.6	0.01
Days since active follicle at NPD reached ≥ 10 mm	4.0 ± 0.3	5.8 ± 0.6	<0.01
P4[4] ≥ 0.5 ng/mL at NPD (%)	83	73	0.38

[1]Treatment = 18 ± 3 d after AI cows were randomly assigned to receive (G25 treatment) or not (NoG25 treatment) GnRH 25 ± 3 d after AI. At 32 ± 3 d after AI, nonpregnant cows from both treatments with at least one CL ≥ 15 mm received PGF2α-24 h-PGF2α-32 h-GnRH-16 to 18 h-TAI. Cows without a CL received a modified Ovsynch protocol with two PGF2α treatments and progesterone supplementation. Blood samples were not available for two cows (one from each treatment).
[2]NPD = nonpregnancy diagnosis.
[3]One cow in the G25 treatment ovulated on Day 21 and after GnRH treatment 25 ± 3 d after AI.
[4]P4 = progesterone

Table 3.4. Ovarian responses and response to resynchronization protocols from the time of nonpregnancy diagnosis to the time of insemination for cows with or without a corpus luteum at nonpregnancy diagnosis that received the G25 and NoG25 treatment.

	Ovarian status at nonpregnancy diagnosis					
	Cows with a CL			Cows without a CL		
Item	G25 (n = 41)	NoG25 (n = 32)	*P*-value	G25 (n = 5)	NoG25 (n = 12)	*P*-value
P4[1] at NPD[2] (ng/mL)	2.4 ± 0.4	3.4 ± 0.4	0.03	0.46 ± 0.1	0.30 ± 0.1	0.28
P4 < 0.5 ng/mL at GnRH before TAI[3] (%)	95	100	0.97	100	100	0.99
Ovulatory follicle size (mm)	17.5 ± 0.4	18.4 ± 0.6	0.15	18.4 ± 0.6	17.7 ± 1.2	0.68
Ovulation after TAI (%)	83	91	0.35	100	92	0.97

[1]P4 = progesterone.
[2]NPD = nonpregnancy diagnosis.
[3]TAI = timed AI.

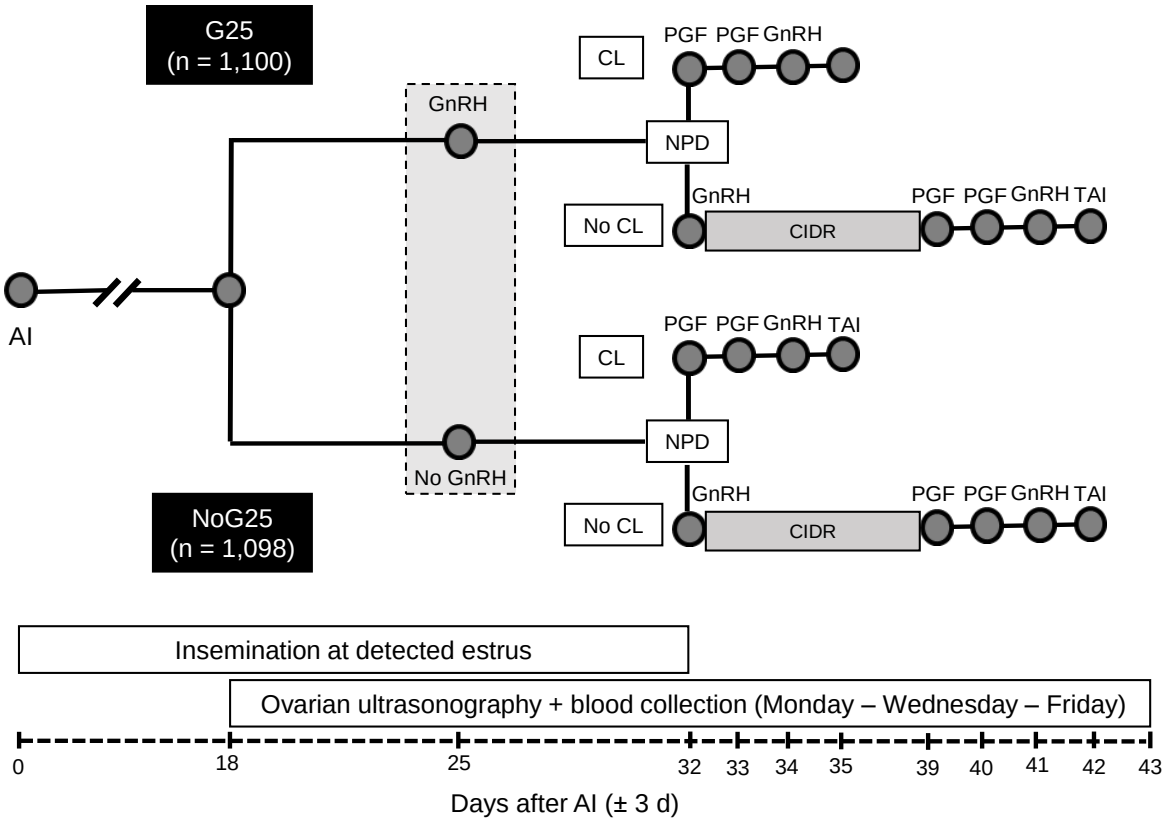

Figure 3.1. Graphical depiction of experimental procedures. Every Monday, cows at 18 ± 3 d after AI (first or second and greater) were stratified by parity (primiparous vs. multiparous) and randomly assigned to receive (G25 treatment; n = 1,100) or not receive (NoG25 treatment; n = 1,098) GnRH treatment 25 ± 3 d after AI. Nonpregnancy diagnosis was performed 32 ± 3 d after AI by transrectal ultrasonography in cows from both treatments not previously re-inseminated in estrus. Nonpregnant cows from both treatments were classified based on the ovarian structures present on their ovaries. Cows with at least one CL ≥ 15 mm and a follicle ≥ 10 mm in diameter (G25 = 325 and NoG25 = 272) received a PGF2α treatment immediately after NPD, a second PGF2α treatment 24 h later, GnRH 32 h after the second PGF2α treatment, and timed AI 16 to 18 h later. Cows that did not meet these criteria (G25 = 53 and NoG25 = 78) were treated with the Ovsynch protocol with two PGF2α treatments and progesterone supplementation (GnRH and CIDR insertion at NPD, CIDR removal and PGF2α 7 d later, PGF2α 1 d later, GnRH 32 h after the second PGF2α, and fixed time AI 16 to 18 h after the GnRH). In a sub-group of cows (G25 = 166 and NoG25 =196), ovarian responses were monitored by transrectal ultrasonography following a Monday, Wednesday, Friday schedule (enrollment and nonpregnancy diagnosis were performed on Mondays) from 18 ± 3 d after AI until cows were either diagnosed pregnant or re-inseminated. At the time of transrectal ultrasonography a blood sample was obtained to measure circulating concentrations of progesterone. NPD = nonpregnancy diagnosis, CL = corpus luteum, TAI = timed AI.

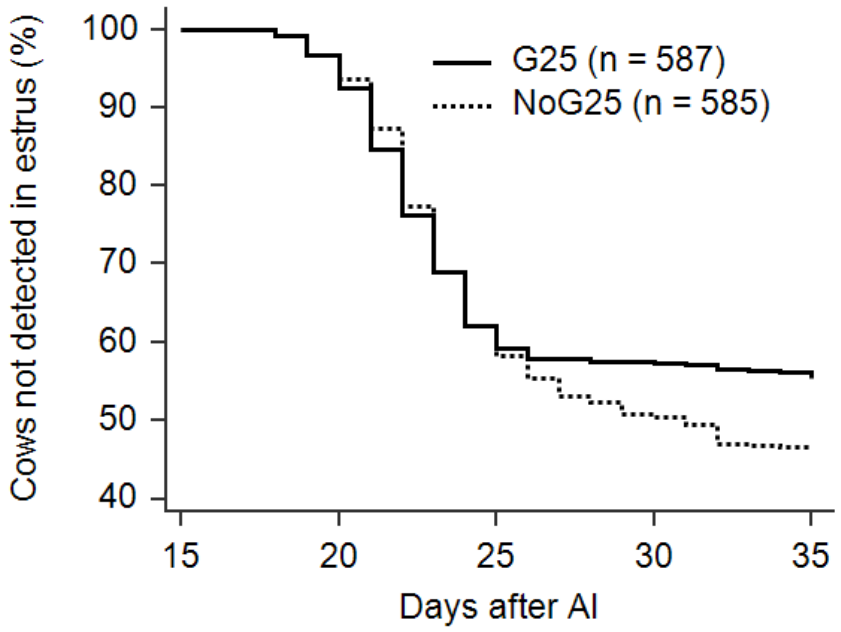

Figure 3.2. Kaplan-Meier survival curves for time to insemination at detected estrus from enrolment (18 ± 3 d after AI) until nonpregnancy diagnosis (32 ± 3 d after AI) for cows in the G25 and NoG25 treatment. The hazard of insemination at detected estrus was greater (P = 0.04) for the NoG25 treatment (hazard ratio = 1.20, 95% CI: 1.01 to 1.43). Median time to re-insemination at detected estrus for the NoG25 treatment was 31 d (95% CI: 27 to 35), whereas it could not be calculated for the G25 treatment because less than 50% of the cows were re-inseminated at detected estrus.

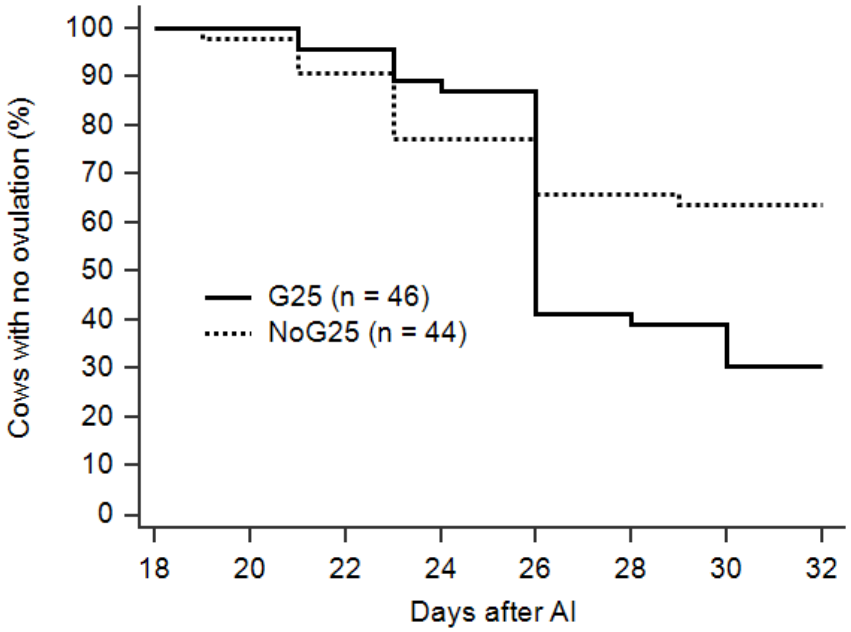

Figure 3.3. Kaplan-Meier survival curves for time to ovulation from enrolment (18 ± 3 d after AI) until nonpregnancy diagnosis (32 ± 3 d after AI) for cows in the G25 and NoG25 treatment. The hazard of ovulation was greater (P = 0.04) for the G25 treatment (hazard ratio = 1.89, 95% CI: 1.05 to 3.5). Median time to ovulation for the G25 treatment was 26 d (95% CI: 26 to 30). Median time to ovulation could not be calculated for the NoG25 treatment because less than 50% of the cows ovulated.

DISCUSSION

In the present experiment we evaluated a management strategy that consisted of removing the first GnRH injection of the Resynch-25 protocol to avoid disrupting estrus expression after the previous insemination while achieving a short interval between TAI services (i.e., 35 ± 3 d). A treatment to increase P/AI of cows with no CL at NPD was also included because these cows were not expected to properly respond to the shortened protocol. In support of our hypotheses, removing the first GnRH of the Resynch-25 protocol affected the pattern of re-insemination and ovarian responses before NPD. Our current data also supported the hypothesis that there would not be a reduction in overall proportion of pregnant cows (combining insemination of cows at detected estrus and TAI) despite a reduction in P/AI for cows with a CL at NPD in the NoG25 treatment.

The different pattern of re-insemination after GnRH 25 ± 3 d after AI and the greater hazard of AI at detected estrus for the NoG25 treatment supported the hypothesis that estrus expression would be limited by GnRH treatment. The reduction in the proportion of cows detected in estrus in the G25 treatment was similar to that observed (~10 percentage points) for cows that received GnRH in the same range of days after AI (Bruno et al., 2014). The most likely mechanism underlying the reduction in estrus expression was lack of an estradiol surge due to the shift from estradiol to progesterone secretion by the granulosa cells of the pre-ovulatory follicle in response to an LH surge (Berndtson et al., 1995; Komar et al., 2001; Jo and Fortune, 2003). Indeed, the pattern of estrus expression after AI clearly reflected the effect of GnRH treatment because it was similar for the two treatments up to 25 d after AI when the vast majority of cows in the G25 treatment received GnRH (data not shown).

The physiological response to treatments from 18 ± 3 to 32 ± 3 d after AI also reflected the effect of GnRH on ovarian responses. The proportion of cows that ovulated in response to GnRH was in agreement with previous experiments (Giordano et al., 2012c; Bruno et al., 2014) and was responsible for the greater proportion of cows with a CL at NPD in the G25 treatment. Interestingly, the difference between treatments was only 12.7 percentage points which may be a reflection of the substantial proportion of cows with a CL formed after the previous AI (data not shown) and the occurrence of spontaneous ovulation before NPD in 36% of the cows in the NoG25 treatment. Other factors may have been responsible for the small difference in the proportion of cows with a CL at NPD between treatments. For example, lack of response to GnRH in cows that did not have a CL formed after the previous AI or cows without spontaneous ovulation from 18 to 32 d after AI in the G25 treatment. For these cows, lack of ovulation in response to GnRH may have been due to the stage of follicle development at the time of GnRH treatment. Indeed, there was no difference in the proportion of cows with an active follicle but cows that ovulated had larger follicles than cows that did not ovulate. It is also possible that ovulation was suppressed by the negative effect of P4 on GnRH-induced LH release (Giordano et al., 2012a; Lima et al., 2013; Stevenson and Pulley, 2016) in cows with a CL formed after the previous AI (data not shown). In some of these cows; however, the CL present at the time of the GnRH injection could have regressed before NPD.

From a practical perspective, the greatest difference resulting from the two strategies compared in this experiment would likely be a different proportion of cows detected in estrus before NPD. This could translate into fewer cows needing resynchronization of ovulation and TAI. Conversely, the greater percentage of cows without a CL at NPD in the NoG25 treatment would lead to more cows needing the Ovsynch protocol with P4 supplementation. For example,

13% of the cows in NoG25 and 9% of the cows in the G25 treatment needed the Ovsynch protocol with P4 supplementation in our experiment. This protocol is more expensive and delays re-insemination by a week. The greater proportion of cows without a CL at NPD in the NoG25 group was also responsible for the similar interbreeding interval observed (i.e., delayed re-insemination of cows with NoCL offset the benefit of more inseminations at detected estrus in the NoG25 treatment). Although the benefits and drawbacks of each program for nonpregnant cows may offset each other, another benefit of the NoG25 treatment would be not giving GnRH to pregnant cows 25 ± 3 d after AI. Removing this treatment would reduce reproductive program costs and labor because of fewer hormonal treatments while avoiding the unnecessary treatment of pregnant cows. Many factors important to the success of the programs tested in this experiment such as estrus detection efficiency, response to GnRH, ovarian responses after a previous AI, and P/AI vary dramatically across farms. Therefore, the choice of management strategy for individual farms could be based on the expected success for each program and/or the preference to inseminate more cows at detected estrus or TAI.

In support of our hypothesis, P/AI for cows with a CL at NPD in the NoG25 treatment was reduced, reflecting the detrimental effect of removing the GnRH treatment 25 ± 3 d after AI. Few experiments evaluated the effect of removing the first GnRH of Ovsynch-type resynchronization protocols in lactating dairy cows. For example, Stevenson and Martel (2009) reported no difference in P/AI when cows received either saline or GnRH 25 ± 3 d after AI (24.8 vs. 27.7%, respectively) but a 50% reduction in P/AI when the same treatments were compared 32 ± 3 d after AI (29.5 vs 19.8%, respectively). Taken together, the results of previous experiments and ours support the notion that the time interval after AI to initiate a shortened protocol (i.e., without the initial GnRH) may be relevant to the fertility of TAI services. Initiating

such protocol at approximately 32 ± 3 d after AI seems optimal because of the high proportion of cows that are at a favorable stage of the estrous cycle at this time point.

Interestingly, P/AI for cows with a CL in the NoG25 treatment was within the range observed for cows resynchronized with Ovsynch (Fricke et al., 2003; Sterry et al., 2006; Bruno et al., 2014) despite removing the GnRH treatment to initiate a new follicular wave during the protocol. The greater P/AI for cows with a CL in the G25 treatment; however, suggests that fertility was suboptimal for cows that did not receive GnRH 25 ± 3 d after AI. This was in spite of the fact that only cows at a favorable stage of the estrous cycle at NPD received the shortened protocol. The most likely explanation for reduced P/AI was the smaller proportion of cows that initiated a new follicular wave 7 d before NPD. Another contributing factor to the reduced P/AI could have been greater variation for time to initiation of the follicular wave. Indeed, the well-documented benefit of ovulation in response to the first GnRH of Ovsynch-type protocols on fertility of TAI (Chebel et al., 2003; Bisinotto et al., 2010; Keskin et al., 2010) has been attributed, at least in part, to better synchronization of follicle development (Pursley et al., 1995; Vasconcelos et al., 1999; Wiltbank et al., 2011). Our data for ovarian responses suggest that for most cows the ovulatory follicle in the NoG25 treatment was older. This observation was supported by the differences in pattern of ovulation before NPD, follicle size at NPD, and the longer period of dominance of the ovulatory follicle. This is relevant because it has been suggested that extending the period of dominance of the ovulatory follicle is detrimental to embryo quality due to asynchrony between timing of oocyte maturation and ovulation (Revah and Butler, 1996). Extending the period of dominance by 2 d, similar to our observations, did not reduce fertilization rate but reduced embryo quality (Cerri et al., 2009). Therefore, we speculate that the longer period of dominance of the ovulatory follicle for cows with a CL in the NoG25

treatment might explain, at least in part, the reduced P/AI for these cows. It is also possible that for some cows the opposite problem reduced fertility to TAI. In this case, cows that ovulated very close to the day of NPD could have had an immature follicle at the time of the GnRH before TAI.

Although the variation in timing of initiation of the new follicular wave is the most likely reason for the difference in P/AI for cows with a CL, we cannot separate or rule out the potential confounding effect of inseminating more cows at detected estrus in the NoG25 treatment. It is possible that some cows inseminated at detected estrus in NoG25 may have conceived if they received TAI. This is supported by the relatively high P/AI for cows inseminated at detected estrus in NoG25 and the similar P/AI for both treatments when combining data for all AI services. On other hand, we explored the potential confounding effect of parity on P/AI because more primiparous than multiparous cows were detected in estrus before NPD. However, the proportion of primiparous cows that had a CL at NPD and received TAI was similar for NoG25 and G25 (~19%), thereby the lower P/AI for the NoG25 treatment could not be attributed to the effect of parity.

Customizing synchronization of ovulation protocols to the ovarian status of cows can reduce the interbreeding interval (current experiment), increase AI at detected estrus (Stevenson et al., 2003, Giordano et al., 2015), and optimize P/AI (Sterry et al., 2006; Bisinotto et al., 2015; Giordano et al., 2016). Differential treatment of cows without a CL at NPD in our experiment ensured substantially greater P/AI than expected if these cows would have received the shortened protocol. Indeed, P/AI for cows with no CL (39.4% for both treatments combined) was relatively high and similar to that observed in other experiments (Giordano et al., 2016). These cows did not benefit from a shorter interbreeding interval; however, greater P/AI because of the Ovsynch

protocol with P4 supplementation was likely more than a shorter interbreeding interval. This is because time to pregnancy is extended more if cows receive TAI with a low chance of conception than if they are re-started in a synchronization of ovulation protocol that leads to greater P/AI.

Adding the second PGF2α before TAI to all protocols in our experiment may explain the relatively high P/AI observed for TAI services. This treatment was added because it has been well-documented that luteal regression in lactating dairy cows is suboptimal (Brusveen et al., 2009; Martins et al., 2011; Giordano et al., 2012d). Therefore, two rather than one PGF2α treatment is more effective to induce complete luteal regression and minimize circulating concentrations of P4 before TAI (Ribeiro et al., 2012; Carvalho et al., 2015; Wiltbank et al., 2015). Also, extended luteal phases and interovulatory intervals are common in lactating dairy cows because of the incidence of embryonic death around the period of maternal recognition of pregnancy (Wijma et al., 2016). Therefore, we expected and observed (data not shown) that some cows would ovulate less than 7 d before NPD. A second PGF2α treatment would be necessary to induce complete luteal regression for these cows with a less mature CL (Henricks et al., 1974; Wiltbank et al., 2015).

CONCLUSIONS

We conclude that removing the first GnRH treatment of the Resynch-25 protocol with two PGF2α treatments for cows with a CL at NPD, and using a modified Ovsynch protocol with P4 supplementation for cows with no CL at NPD allowed insemination of a greater proportion of cows at a detected estrus but did not affect the overall proportion of pregnant cows. Nevertheless, P/AI for cows with a CL at NPD was reduced in cows that did not receive GnRH

25 ± 3 d after AI. The most likely reason for reduced P/AI was more variation in timing of initiation of the follicular wave that gave rise to the ovulatory follicle. Nevertheless, because of differences in detection of estrus before NPD we could not separate or rule out the potential effect of having differences in the population of cows that received TAI.

Additional research is needed to determine the impact of the treatments tested in this experiment on time to pregnancy during lactation and profitability of dairy herds.

ACKNOWLEDGMENTS

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REFERENCES

Abramson, J. H. 2011. WINPEPI updated: computer programs for epidemiologists, and their teaching potential. Epidemiologic Perspectives & Innovations 8:1.

Berndtson, A., S. Vincent, and J. Fortune. 1995. Low and high concentrations of gonadotropins differentially regulate hormone production by theca interna and granulosa cells from bovine preovulatory follicles. Biology of reproduction 52:1334-1342.

Bisinotto, R., R. Chebel, and J. Santos. 2010. Follicular wave of the ovulatory follicle and not cyclic status influences fertility of dairy cows. Journal of dairy science 93:3578-3587.

Bisinotto, R., I. Lean, W. Thatcher, and J. Santos. 2015. Meta-analysis of progesterone supplementation during timed artificial insemination programs in dairy cows. Journal of dairy science 98:2472-2487.

Bruno, R., J. Moraes, J. Hernández-Rivera, K. Lager, P. Silva, A. Scanavez, L. Mendonça, R. Chebel, and T. Bilby. 2014. Effect of an Ovsynch56 protocol initiated at different intervals after insemination with or without a presynchronizing injection of gonadotropin-releasing hormone on fertility in lactating dairy cows. Journal of dairy science 97:185-194.

Brusveen, D., A. Souza, and M. Wiltbank. 2009. Effects of additional prostaglandin F 2α and estradiol-17β during Ovsynch in lactating dairy cows. Journal of dairy science 92:1412-1422.

Caraviello, D., K. Weigel, P. Fricke, M. Wiltbank, M. Florent, N. Cook, K. Nordlund, N. Zwald, and C. Rawson. 2006. Survey of management practices on reproductive performance of dairy cattle on large US commercial farms. Journal of dairy science 89:4723-4735.

Carvalho, P., M. Fuenzalida, A. Ricci, A. Souza, R. Barletta, M. Wiltbank, and P. Fricke. 2015. Modifications to Ovsynch improve fertility during resynchronization: Evaluation of presynchronization with gonadotropin-releasing hormone 6 d before initiation of Ovsynch and addition of a second prostaglandin F2α treatment. Journal of dairy science 98:8741-8752.

Cerri, R. L., H. M. Rutigliano, R. C. Chebel, and J. E. Santos. 2009. Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. Reproduction 137:813-823.

Chebel, R. C., J. E. Santos, R. L. Cerri, K. N. Galvão, S. O. Juchem, and W. W. Thatcher. 2003. Effect of resynchronization with GnRH on day 21 after artificial insemination on pregnancy rate and pregnancy loss in lactating dairy cows. Theriogenology 60:1389-1399.

Ferguson, J. D. and A. Skidmore. 2013. Reproductive performance in a select sample of dairy herds. Journal of dairy science 96:1269-1289.

Fricke, P., D. Caraviello, K. Weigel, and M. Welle. 2003. Fertility of dairy cows after resynchronization of ovulation at three intervals following first timed insemination. Journal of dairy science 86:3941-3950.

Galvão, K., P. Federico, A. De Vries, and G. M. Schuenemann. 2013. Economic comparison of reproductive programs for dairy herds using estrus detection, timed artificial insemination, or a combination. Journal of dairy science 96:2681-2693.

Galvão, K., J. Santos, R. Cerri, R. Chebel, H. Rutigliano, R. Bruno, and R. Bicalho. 2007. Evaluation of methods of resynchronization for insemination in cows of unknown pregnancy status. Journal of dairy science 90:4240-4252.

Giordano, J., P. Fricke, and V. Cabrera. 2013. Economics of resynchronization strategies including chemical tests to identify nonpregnant cows. Journal of dairy science 96:949-961.

Giordano, J., P. Fricke, J. Guenther, G. Lopes, M. Herlihy, A. Nascimento, and M. Wiltbank. 2012a. Effect of progesterone on magnitude of the luteinizing hormone surge induced by two different doses of gonadotropin-releasing hormone in lactating dairy cows. Journal of dairy science 95:3781-3793.

Giordano, J., A. Kalantari, P. Fricke, M. Wiltbank, and V. Cabrera. 2012b. A daily herd Markov-chain model to study the reproductive and economic impact of reproductive programs combining timed artificial insemination and estrus detection. Journal of dairy science 95:5442-5460.

Giordano, J., M. Stangaferro, R. Wijma, W. Chandler, and R. Watters. 2015. Reproductive performance of dairy cows managed with a program aimed at increasing insemination of cows in estrus based on increased physical activity and fertility of timed artificial inseminations. Journal of dairy science 98:2488-2501.

Giordano, J., M. Thomas, G. Catucuamba, M. Curler, M. Masello, M. Stangaferro, and R. Wijma. 2016. Reproductive management strategies to improve the fertility of cows with a suboptimal response to resynchronization of ovulation. Journal of dairy science 99:2967-2978.

Giordano, J., M. Wiltbank, J. Guenther, M. Ares, G. Lopes, M. Herlihy, and P. Fricke. 2012c. Effect of presynchronization with human chorionic gonadotropin or gonadotropin-releasing hormone 7 days before resynchronization of ovulation on fertility in lactating dairy cows. Journal of dairy science 95:5612-5625.

Giordano, J., M. Wiltbank, J. Guenther, R. Pawlisch, S. Bas, A. Cunha, and P. Fricke. 2012d. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. Journal of dairy science 95:639-653.

Henricks, D., J. Long, J. Hill, and J. Dickey. 1974. The effect of prostaglandin F2α during various stages of the oestrous cycle of beef heifers. Journal of reproduction and fertility 41:113-120.

Jo, M. and J. Fortune. 2003. Changes in oxytocin receptor in bovine preovulatory follicles between the gonadotropin surge and ovulation. Molecular and cellular endocrinology 200:31-43.

Keskin, A., G. Yilmazbas-Mecitoglu, A. Gumen, E. Karakaya, R. Darici, and H. Okut. 2010. Effect of hCG vs. GnRH at the beginning of the Ovsynch on first ovulation and conception rates in cyclic lactating dairy cows. Theriogenology 74:602-607.

Komar, C. M., A. K. Berndtson, A. C. Evans, and J. E. Fortune. 2001. Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA for p450 aromatase and p450 17alpha-hydroxylase, in bovine preovulatory follicles. Biol Reprod 64:1797-1805.

Lima, F., E. Ribeiro, R. Bisinotto, L. Greco, N. Martinez, M. Amstalden, W. Thatcher, and J. Santos. 2013. Hormonal manipulations in the 5-day timed artificial insemination protocol to optimize estrous cycle synchrony and fertility in dairy heifers. Journal of dairy science 96:7054-7065.

Lopes, G., J. Giordano, A. Valenza, M. Herlihy, J. Guenther, M. Wiltbank, and P. Fricke. 2013. Effect of timing of initiation of resynchronization and presynchronization with gonadotropin-releasing hormone on fertility of resynchronized inseminations in lactating dairy cows. Journal of dairy science 96:3788-3798.

Martins, J., R. Policelli, L. Neuder, W. Raphael, and J. Pursley. 2011. Effects of Cloprostenol sodium at final prostaglandin F 2α of Ovsynch on complete luteolysis and pregnancy per artificial insemination in lactating dairy cows. Journal of dairy science 94:2815-2824.

Mendonça, L. G., S. T. Dewey, G. Lopes, F. A. Rivera, F. S. Guagnini, J. P. Fetrow, T. R. Bilby, and R. C. Chebel. 2012. Effects of resynchronization strategies for lactating Holstein cows on pattern of reinsemination, fertility, and economic outcome. Theriogenology 77:1151-1158.

Moreira, F., C. Orlandi, C. Risco, R. Mattos, F. Lopes, and W. Thatcher. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. Journal of Dairy Science 84:1646-1659.

Pursley, J., M. Mee, and M. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF 2α and GnRH. Theriogenology 44:915-923.

Revah, I. and W. Butler. 1996. Prolonged dominance of follicles and reduced viability of bovine oocytes. Journal of reproduction and fertility 106:39-47.

Ribeiro, E., R. Bisinotto, M. Favoreto, L. Martins, R. Cerri, F. Silvestre, L. Greco, W. Thatcher, and J. Santos. 2012. Fertility in dairy cows following presynchronization and administering twice the luteolytic dose of prostaglandin F 2α as one or two injections in the 5-day timed artificial insemination protocol. Theriogenology 78:273-284.

Rutigliano, H., F. Lima, R. Cerri, L. Greco, J. Vilela, V. Magalhães, F. Silvestre, W. Thatcher, and J. Santos. 2008. Effects of method of presynchronization and source of selenium on uterine health and reproduction in dairy cows. Journal of dairy science 91:3323-3336.

Scott, B. D. 2016. Incorporation of dairy farm survey data and epidemiological patterns for agent-based simulation modeling of dairy herd dynamics. MS Thesis. Cornell University.

Sterry, R., M. Welle, and P. Fricke. 2006. Effect of interval from timed artificial insemination to initiation of resynchronization of ovulation on fertility of lactating dairy cows. Journal of dairy science 89:2099-2109.

Stevenson, J. and C. Martel. 2009. Resynchronized ovulation in lactating dairy cattle of unknown pregnancy: Occurrence and timing of gonadotropin-releasing hormone. The Professional Animal Scientist 25:605-609.

Stevenson, J. S., J. A. Cartmill, B. A. Hensley, and S. Z. El-Zarkouny. 2003. Conception rates of dairy cows following early not-pregnant diagnosis by ultrasonography and subsequent treatments with shortened Ovsynch protocol. Theriogenology 60:475-483.

Stevenson, J. S. and S. L. Pulley. 2016. Feedback effects of estradiol and progesterone on ovulation and fertility of dairy cows after gonadotropin-releasing hormone-induced release of luteinizing hormone. J Dairy Sci 99:3003-3015.

Vasconcelos, J., R. Silcox, G. Rosa, J. Pursley, and M. Wiltbank. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology 52:1067-1078.

Wijma, R., M. Stangaferro, and J. Giordano. 2015. Characterization of ovarian function in nonpregnant previously inseminated lactating dairy cows. J. Dairy. Sci. 98 E-supplement 1.

Wijma, R., M. L. Stangaferro, M. M. Kamat, S. Vasudevan, T. L. Ott, and J. O. Giordano. 2016. Embryo Mortality Around the Period of Maintenance of the Corpus Luteum Causes Alterations to the Ovarian Function of Lactating Dairy Cows 1. Biology of Reproduction 95:Article 112, 111-114.

Wiltbank, M., R. Sartori, M. Herlihy, J. L. M. Vasconcelos, A. Nascimento, A. Souza, H. Ayres, A. Cunha, A. Keskin, and J. Guenther. 2011. Managing the dominant follicle in lactating dairy cows. Theriogenology 76:1568-1582.

Wiltbank, M. C., G. M. Baez, F. Cochrane, R. V. Barletta, C. R. Trayford, and R. T. Joseph. 2015. Effect of a second treatment with prostaglandin F2α during the Ovsynch protocol on luteolysis and pregnancy in dairy cows. Journal of dairy science 98:8644-8654.

CHAPTER IV

A SHORT RESYNCHRONIZATION PROTOCOL BASED ON OVARIAN STRUCTURES PRESENT AT NONPREGNANCY DIAGNOSIS REDUCED TIME TO PREGNANCY IN LACTATING DAIRY COWS

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ABSTRACT

Our objectives were to evaluate time to pregnancy after the first service postpartum and pregnancy/AI (P/AI) in dairy cows managed with two resynchronization of ovulation programs. After first service, lactating Holstein cows were blocked by parity (primiparous vs. multiparous) and randomly assigned to the Day 32-Resynch (R32; n = 1,010) or Short-Resynch (SR; n = 1,000) treatments. Nonpregnancy diagnosis (NPD) was conducted 32 ± 3 d after AI by transrectal ultrasonography (TUS). Nonpregnant cows in R32 received the Ovsynch protocol [GnRH-7 d-PGF2α-56 h-GnRH-16 to 18 h-timed AI (TAI)]. Cows in SR with a corpus luteum (CL) ≥15 mm and a follicle ≥10 mm at NPD received PGF2α, PGF2α 24 h later, GnRH 32 h later, and TAI 16 to 18 h later. Cows in SR without a CL ≥15 mm and/or a follicle ≥10 mm at NPD received a modified Ovsynch protocol with two PGF2α treatments and progesterone (P4) supplementation (GnRH+CIDR-7 d-CIDR removal+PGF2α-24 h-PGF2α-32 h-GnRH-16 to 18 h-TAI). Blood samples were collected from a subgroup of cows at NPD (R32 = 143 and SR = 204), and GnRH before TAI (R32 = 114 and SR = 121) to measure P4 concentration. Binomial outcomes were analyzed with logistic regression and hazard of pregnancy (R32 = 485 and SR = 462) with Cox's proportional regression in SAS. For P/AI analysis, the TAI service was the

experimental unit (R32 = 615 and SR = 748). Models included treatment and parity as fixed effects and farm as random effect. The hazard of pregnancy was greater for the SR treatment (hazard ratio = 1.18, 95% CI: 1.01 to 1.37). Median time to pregnancy was 95 and 79 d for the R32 and SR treatments, respectively. At NPD, 71.4 and 72.9% of cows had a CL for the R32 and SR treatments, respectively. Treatment did not affect overall P/AI 32 ± 3 d after AI (R32 = 29.6% vs. SR = 33.0%), or for cows with a CL at NPD (R32 = 31.9% vs. SR = 31.6%). For cows with no CL at NPD, P/AI was greater for the SR (37.0%) than the R32 (23.9%) treatment. Pregnancy loss from 32 to 63 d after AI was similar for all services combined (R32 = 7.3% vs. SR = 10.9%), and for cows with no CL at NPD (R32 = 12.2% vs. SR = 8.5%) but tended to be affected by treatment for cows with a CL at NPD (R32 = 5.8% vs. SR = 12.0%). Treatment affected the proportion of cows with P4 ≤ 0.5 ng/mL at the GnRH before TAI for all cows (R32 = 68.4% vs. SR = 81.8%), tended to have an effect among cows with a CL (R32 = 70.0% vs. SR = 81.8%), and had no effect for cows with no CL (R32 = 64.7% vs. SR = 81.8%). We concluded that the SR program reduced time to pregnancy because of a reduction of the interbreeding interval for cows with a CL at NPD and greater P/AI in cows with no CL at NPD.

INTRODUCTION

Dairy farm profitability depends on the reproductive performance of lactating dairy cows which is primarily determined by the rate at which cows become pregnant after the end of the voluntary waiting period (Louca and Legates, 1968; Oltenacu et al., 1981). In spite of recent gains on first service P/AI due to improved dairy herd management and the adoption of synchronization of ovulation protocols (Wiltbank and Pursley, 2014), a substantial proportion of lactating dairy cows remain nonpregnant after the first service and need immediate re-

insemination. Therefore, to minimize the interbreeding interval many farms use reproductive management strategies for second and greater AI services that combine insemination at detected estrus and timed AI (TAI) after resynchronization of ovulation with Ovsynch-type protocols [(Pursley et al., 1995); commonly referred to as Resynch]. These protocols are usually initiated at the time of or seven days before nonpregnancy diagnosis [NPD; (Fricke et al., 2003; Bartolome et al., 2005; Giordano et al., 2012c)].

Farms that combine AI at detected estrus and TAI can initiate Resynch as early as 25 ± 3 d after AI. Although this strategy can be beneficial because of the relatively short interbreeding interval for cows that receive TAI (i.e., 35 ± 3 d), the first GnRH treatment of the protocol coincides with the time at which many cows are expected to display estrus (Remnant et al., 2015; Wijma et al., 2017). The GnRH-induced LH surge reduces the proportion of cows displaying natural estrus (Mendonça et al., 2012; Bruno et al., 2014; Wijma et al., 2017) through induction of ovulation and/or by suppressing the estradiol surge responsible for estrous behavior (Berndtson et al., 1995; Komar et al., 2001; Jo and Fortune, 2003). Further, the pregnancy status of cows at the time of the GnRH treatment 25 ± 3 d is unknown because none of the currently available methods for pregnancy testing for dairy cows allows accurate pregnancy detection at or before 25 ± 3 d after AI. Therefore, a substantial proportion of cows receive an unnecessary treatment because they are pregnant; increasing treatment costs, labor costs, and in some cases disrupting cow normal resting behavior.

Thus, to take advantage of a short interbreeding interval for TAI services while avoiding a reduction in the proportion of cows AI at detected estrus before NPD and unnecessary treatment of pregnant cows with GnRH, we recently conducted a proof of concept experiment to evaluate a reproductive management strategy based on the ovarian structures present at NPD 32

± 3 d after AI (Wijma et al., 2017). Cows with a CL ≥ 15 mm and follicle ≥ 10 mm at NPD (hereafter CL cows) received a resynchronization of ovulation protocol without an initial GnRH treatment to induce a new follicular wave (Short-Resynch; PGF2α, 1 d later PGF2α, 32 h later GnRH, and TAI 16 to 18 h after GnRH). On the other hand, cows not expected to respond to the Short-Resynch protocol because of their ovarian status (i.e., no CL ≥15 mm and/or no follicle ≥10 mm; hereafter no CL cows) received a modified Ovsynch protocol with progesterone (P4) supplementation (GnRH + P4 intravaginal device-7 d-PGF2α + P4 intravaginal device removal-1 d-PGF2α-32 h-GnRH-16 to 18 h-TAI). This management strategy was compared to a similar program in which all cows received GnRH treatment 7 d before NPD (i.e., 25 ± 3 d after AI). Removing the GnRH treatment 25 ± 3 d after AI resulted in ~17% more cows inseminated at detected estrus (GnRH = 44.6% and NoGnRH = 53.5%) but it also resulted in a P/AI reduction of ~ 8 percentage points for TAI services in CL cows (GnRH = 40.6% and NoGnRH = 32.8%). Nevertheless, the cumulative proportion of cows pregnant after AI at detected estrus and TAI was similar for both groups (GnRH = 39.6% and NoGnRH = 39.0%) because of the greater number of pregnancies generated through insemination of cows at detected estrus before NPD in the Short-Resynch treatment. Although results from this experiment (Wijma et al., 2017) were promising, additional research is needed to determine if the strategy based on ovarian status at NPD is superior to traditional programs combing AI at detected estrus and TAI after blanket use of resynchronization of ovulation with the Resynch protocol. Moreover, additional data is necessary to corroborate that removal of the initial GnRH treatment does not compromise P/AI for cows with CL at NPD to an extent that may offset the benefit of shorter interbreeding interval for TAI services.

Thus, we hypothesized that a resynchronization program based on ovarian structures present at the time of NPD (hereafter referred to as Short-Resynch) would reduce time to pregnancy after the first service when compared with blanket use of TAI after the D32-Resynch protocol. Time to pregnancy would be reduced because of the shorter interbreeding interval for CL cows and increased P/AI for no CL cows. Therefore, the objectives of this experiment were to evaluate the effect of Short-Resynch on time to pregnancy after the first service, P/AI, and physiological outcomes before TAI.

MATERIALS AND METHODS

This experiment was conducted from February 2016 to May of 2017 in two commercial dairy farms located in Tompkins and Cayuga counties in New York State. All procedures were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at Cornell University.

In both farms, cows were housed in free stall barns and were fed a total mixed ration once a day with ad libitum access to feed and water. Farm A milked ~1,300 cows with average milk yield of ~43 kg/d. Cows were milked thrice daily at approximately 8 h intervals until February 2017 when milking frequency changed to four times per day at approximately 6 h intervals. All cows received recombinant bovine somatotropin (500 mg of Sometribove zinc; Posilac, Elanco Animal Health, Indianapolis, IN) at 10 or 11 d intervals beginning at 80 ± 3 DIM in primiparous cows and 110 ± 3 DIM in multiparous cows until dry off. Primiparous cows received first service at 82 ± 3 DIM and multiparous cows at 67 ± 3 DIM after synchronization of ovulation with the Double-Ovsynch protocol (Souza et al., 2008). Farm B milked ~1,900 cows thrice daily at approximately 8 h intervals and had average milk yield per cow of ~42 kg/d. All cows received

recombinant bovine somatotropin at 14 d intervals beginning at 60 ± 3 DIM until dry off. Primiparous and multiparous cows where synchronized with the Presynch-Ovsynch protocol (Moreira et al., 2001). Cows were eligible to receive AI after the first and second PGF2α treatments of Presynch-Ovsynch at 53 ± 3 and 67 ± 3 DIM, whereas cows not detected in estrus received TAI at 79 ± 3 DIM. During the experiment, 86.5% (1,103/1,274) of the cows enrolled received the first service at detected estrus whereas the remaining 13.4% (171/1274) of the cows received TAI.

Every week, cows that received a previous AI service were blocked by parity (primiparous vs. multiparous) and randomly assigned to the D32-Resynch (R32) or Short Resynch (SR) treatment. Cows remained in the same treatment until the end of the experiment. Cows that received their first service postpartum at or after the beginning of the experiment (R32 = 1,010 and SR = 1,000) were included for the evaluation of time to pregnancy. Conversely, cows that had already received their first service before the beginning of the experiment were enrolled but only data from individual TAI services were collected (R32 = 207 and SR = 270) for subsequent analysis of P/AI and pregnancy loss. All cows detected in estrus after AI were immediately inseminated. In farm A, detection of estrus was conducted using a combination of visual observation and physical activity monitoring with leg-mounted activity tags (Afi-ActII, Afikim, Kibbutz Afikim, Israel), whereas in Farm B detection of estrus was conducted through visual observation and tail paint removal. Nonpregnancy diagnosis was performed by transrectal ultrasonography (TUS; Ibex Pro, Ibex, Loveland, CO) 32 ± 3 d after AI in cows from both experimental treatments not previously re-inseminated at detected estrus. In nonpregnant cows, size of follicles and corpora lutea present at NPD was estimated using the ultrasound machine goggle's screen grid lines comprising squares of 10 mm by 10 mm. Pregnant cows received no

further treatment. All TUS examinations were conducted by veterinarians. In farm A, the veterinarian was one of the co-authors, whereas in farm B it was one of the co-authors and the practicing veterinarian.

Nonpregnant cows from the SR experimental treatment were classified and then treated based on the ovarian structures present at NPD (Figure 4.1). Cows with at least one CL ≥ 15 mm and at least one ovarian follicle ≥ 10 mm in diameter (CL cows) received PGF2α (500 μg of Cloprostenol Sodium, Estrumate, Merck Animal Health, Summit, NJ, USA) immediately after NPD, a second PGF2α 24 h later, GnRH (100 μg of Gonadorelin diacetate tetrahydrate, Fertagyl, Merck Animal Health, Summit, NJ, USA) 32 h after the second PGF2α, and TAI 16 to 18 h later (Figure 4.1). Cows that did not meet these criteria (No CL cows) received a modified Ovsynch protocol with two PGF2α treatments and P4 supplementation through an intravaginal P4 releasing device (1.38 g of Progesterone, Eazi-Breed CIDR, Zoetis, Florham Park, NJ) from the time of the first GnRH to the first PGF2α treatment of the protocol (GnRH + CIDR-7 d-CIDR removal + PGF2α-24 h-PGF2α-32 h-GnRH-16 to 18 h-TAI; Figure 4.1). Cows detected in estrus after NPD and before TAI were immediately inseminated. Every time a cow in the SR treatment was diagnosed nonpregnant was classified as CL or No CL cow and received the stipulated treatment based on ovarian status.

Nonpregnant cows in the R32 treatment were immediately enrolled in the D32-Resynch protocol (32 ± 3 d after AI GnRH-7 d-PGF2α-56 h-GnRH-16 to 18 h-TAI; Figure 4.1) to receive TAI. A combination of AI at detected estrus and TAI after the D32-Resynch protocol without differential treatment based on ovarian structures was selected as control treatment because it is one of the most commonly used strategies to manage second and greater AI services in lactating dairy cows in the U.S. (Caraviello et al., 2006; Ferguson and Skidmore, 2013; Scott, 2016).

Reconfirmation of pregnancy was performed 63 ± 3 d after AI by TUS in both farms. A cow was considered to have undergone pregnancy loss when confirmed pregnant at the initial examination and nonpregnant at the time of reconfirmation. Cows with an insemination after a detected estrus between the two pregnancy examinations were also considered to have suffered pregnancy loss. Cows not pregnant at pregnancy reconfirmation were immediately enrolled in the Ovsynch protocol. If they failed to conceive to the insemination immediately after detection of pregnancy loss, cows received the treatments stipulated per their experimental treatment (i.e., R32 or SR).

Transrectal Ultrasonography and Blood Sample Collection for Monitoring Ovarian Responses

Ovarian responses were monitored at the time of NPD and the GnRH treatment before TAI in a subgroup of cows from each treatment (153 and 146 in R32 and SR, respectively) in Farm A. Transrectal ultrasonography was conducted to record size (diameter) of the largest follicle and corpora lutea present on the ovaries. Size of follicles and corpora lutea was estimated using the ultrasound machine (same used for NPD) goggle screen grid lines comprising squares of 10 mm by 10 mm. If the largest follicle was greater than 25 mm and thereby, a potential follicular cyst, the second largest follicle was considered the dominant follicle for analysis. From the same group of cows, blood samples were collected to determine circulating concentration of P4. Blood samples were collected using 8-mL heparinized evacuated tubes (Vacutainer, BD, Franklin Lakes, NJ) via puncture of the coccygeal vein or artery. Samples were placed in a cooler with ice until transported to the laboratory within ≤ 4 h of collection. Samples were centrifuged at 1,700 x g for 20 minutes at 4°C. Plasma aliquots were harvested and transferred to vials for storage at -20°C until assays were performed. Progesterone concentration data at NPD

was available for 205 cows with a CL ≥15 mm and 78 cows with no CL ≥ 15 mm at NPD from both treatments. Data from ovarian structures and P4 concentration at the GnRH treatment before TAI was available for 114 and 121 cows from the R32 and SR treatments, respectively. The proportion of cows with a functional CL based on circulating concentration of P4 (P4 ≥ 1 ng/mL) was the outcome of interest at NPD. At the time of the GnRH injection before TAI outcomes of interest were (1) the proportion of cows with low P4 based on a cutoff value of ≤ 0.5 ng/mL or 1 ng/mL and (2) size of the largest ovarian follicle present.

Determination of Progesterone Concentration

Concentration of P4 in plasma was determined in duplicate with a commercial solid-phase, no-extraction radioimmunoassay (ImmuChem Coated Tube, MP Biomedicals, Costa Mesa, CA). To assess precision of the assays, control samples with high (5.8 ng/mL) and low (0.3 ng/mL) concentrations of P4 were included at the beginning and end of each assay (n = 6 assays). Average detection limit for the P4 assay was 0.1 ng/mL. Average intra-assay CV for the high-concentration sample was 9.7% whereas the inter-assay CV was 13.4%. For the low-concentration sample the average intra-assay CV was 24.2% whereas the inter-assay CV was 31.3%.

Statistical Analysis

This experiment was conducted as a complete randomized block design using parity (primiparous vs. multiparous) as blocking factor. According to sample size calculations conducted using the sample size calculation option of WinPepi version 11.51 (Abramson, 2011), a total of 398 cows per treatment were needed to detect a hazard ratio for pregnancy of 1.25 with

an average probability of survival at the end of the experimental period of 20%, with probability of type I error rate of 5%, and probability of type II error rate of 20%.

Cox's proportional hazards analysis for pregnancy after the first service postpartum was conducted using the PHREG procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) with treatment, parity, and milk production tercile (calculated for farm and parity group within farm) as fixed effects, and farm as random effect. Milk production level was removed from the final model because $P > 0.10$. Only cows that did not conceive to first AI and had at least 210 d at risk of becoming pregnant after the first service were included in the analysis. A cow was considered pregnant for the analysis of time to pregnancy only if it was reconfirmed pregnant 63 ± 3 d after AI. Cows that became not eligible for AI due to a farm management decision (i.e., coded as do not breed), or left the herd due to sale or death were right censored. Kaplan-Meier survival curves were generated to illustrate the rate of pregnancy after the first service postpartum using the survival analysis option of MedCalc (version 12.5.0.0; MedCalc Software bvba, Ostend, Belgium).

Binary outcomes (i.e., P/AI, pregnancy loss, proportion of cows inseminated at detected estrus, proportion of cows not pregnant 210 d after the first service, and proportion of cows with high or low P4 concentration at NPD and the GnRH before TAI) were analyzed using logistic regression with the GLIMMIX procedure of SAS. Treatment and parity (primiparous vs. multiparous) were included as fixed effects and farm as random effect. Days in milk at insemination (≤100, >100 and ≤ 150, >150 and ≤ 200, and >200 days) and season of insemination [warm (June, July, and August) vs. cold (September to May)] were offered to the initial models for P/AI and pregnancy loss. Season was removed from all final models and DIM from the pregnancy loss models because $P > 0.10$.

The level of agreement between TUS and P4 concentration (reference test) to detect the presence of an active CL was determined through calculation of the kappa value for inter-rater agreement obtained with the FREQ procedure of SAS.

Size of the largest ovarian follicle at the time of GnRH before TAI was analyzed by ANOVA using the MIXED procedure of SAS including treatment and parity as fixed effects.

All proportions reported were generated using the FREQ procedure of SAS whereas values for quantitative outcomes are reported as arithmetic means calculated using the MEANS procedure of SAS. All explanatory variables were considered significant if $P \leq 0.05$ whereas a $P > 0.05$ and ≤ 0.10 was considered a tendency.

RESULTS

Time to Pregnancy after First Service

In total, 485 and 462 cows from the R32 and SR treatments, respectively were not pregnant after the first service postpartum and were at least 210 days at risk of becoming pregnant after the first service postpartum unless left the herd due to sale or death. The proportion of second and greater AI services that occurred after estrus detection [$P = 0.56$; R32 = 66.4% (739/1,113) vs. SR = 67.7% (594/878)] and P/AI for these services [$P = 0.26$; R32 = 34.4% (277/805) vs. SR = 37.2% (245/658)] was similar for both treatments.

The hazard of pregnancy was greater ($P = 0.03$) for the SR than the R32 treatment [hazard ratio (HR) = 1.18, 95% CI: 1.01 to 1.37; Figure 4.2] and was similar ($P = 0.15$) for primiparous and multiparous cows [HR = 1.12, 95% CI: 0.96 to 1.31]. Median time to pregnancy was 95 (95% CI: 84 to 108) and 79 d (95% CI: 71 to 96) for the R32 and SR treatment, respectively. Mean time to pregnancy was 111 ± 3 and 100 ± 3 d for the R32 and SR treatment,

respectively. At 210 d after first service, a greater ($P < 0.02$) proportion of cows were not pregnant in the R32 (29.3%; 142/485) than the SR treatment (22.3%; 103/462) and a greater ($P < 0.01$) proportion of multiparous (29.3%; 177/605) than primiparous (19.9%; 68/342) cows were not pregnant.

Pregnancy per Artificial Insemination and Pregnancy Loss

The proportion of cows inseminated at detected estrus after NPD and before TAI was greater for the R32 treatment for all cows [$P < 0.001$; R32 = 14.7%; (108/734) and SR = 8.7%; (71/819)] and cows with a CL at NPD [$P < 0.001$; R32 = 14.6%; (77/527) and SR = 6.5%; (38/583)]. No effect of treatment ($P = 0.68$) was observed for cows with no CL at NPD [R32 = 15.3%; (34/221) and SR = 14.0%; (33/236)]. For the R32 treatment, 50.9% of the AI services at detected estrus were conducted from the time of the first GnRH until the PGF2α injection of the protocol whereas the remaining 49.1% were conducted after the PGF2α and before the morning of TAI. Treatment did not affect P/AI ($P = 0.54$) for AI services that occurred before TAI [R32 = 38.9% (42/108) and SR = 43.7% (31/71)]. These services were not included in the analyses for P/AI and pregnancy loss for TAI services. At NPD the proportion of CL cows was 71.4% (439/615) for R32 and 72.9% (545/748) for the SR treatment. The rest of the cows were classified as No CL cows in both treatment groups.

Overall, P/AI at 32 ± 3 d after TAI was not affected by treatment ($P = 0.14$; Table 4.1) or parity [$P = 0.41$; primiparous = 32.3% (181/561) vs. multiparous = 30.9% (248/802)]. Among cows with a CL at NPD, P/AI was not affected ($P = 0.94$) by treatment (Table 4.1) and tended ($P = 0.09$) to be affected by parity [primiparous = 34.4% (138/401) vs. multiparous = 29.8% (174/583)]. For cows with no CL at NPD, P/AI was greater ($P < 0.01$) for the SR than for the

R32 treatment (Table 4.1), but there was no effect of parity [$P = 0.31$; primiparous = 26.9% (43/160) vs. multiparous = 33.8% (74/219)]. Days in milk at TAI affected P/AI for all AI services ($P = 0.02$) and for AI services in cows with no CL at NPD ($P < 0.001$) whereby, cows with >200 DIM had reduced ($P < 0.05$) P/AI when compared with cows inseminated at earlier DIM. On the other hand, DIM at insemination did not affect ($P = 0.43$) P/AI in cows with a CL at NPD.

Overall P/AI at 63 ± 3 d after TAI was not affected by treatment ($P = 0.62$; Table 4.1) or parity [$P = 0.50$; primiparous = 28.2% (156/553) vs. multiparous = 27.0% (213/788)]. Among cows with a CL at NPD there was no effect of treatment [$P = 0.27$; R32 = 29.6% (129/436) vs. SR = 26.2% (139/531)] or parity [$P = 0.11$; primiparous = 30.4% (120/395) vs. multiparous = 25.9% (148/572)] on P/AI. For cows with no CL at NPD, P/AI was greater ($P < 0.01$) for the SR (32.7%; 65/199) than for the R32 treatment (20.6%; 36/175) but, there was no effect of parity [$P = 0.24$; primiparous = 22.8% (36/158) vs. multiparous = 30.1% (65/216)]. Days in milk at TAI affected P/AI 63 ± 3 d after AI for all AI services ($P = 0.02$) and for cows with no CL at NPD ($P < 0.001$) because cows with DIM >200 had reduced ($P < 0.05$) P/AI when compared with cows inseminated at earlier DIM. On the other hand, DIM did not affect ($P = 0.70$) P/AI for cows with a CL at NPD.

Pregnancy loss for all AI services combined was similar for both treatments ($P = 0.17$; Table 4.1) and was not affected by parity [$P = 0.76$; primiparous = 9.8% (17/173) vs. multiparous = 9.0% (21/234)]. For cows with a CL at NPD, pregnancy loss tended ($P = 0.06$) to be greater for the SR (12.0%; 19/158) than for the R32 (5.8%; 8/137) treatment but there was no effect of parity [$P = 0.95$; primiparous = 9.1% (12/132) vs. multiparous = 9.2% (15/163)]. For cows with no CL at NPD, treatment ($P = 0.59$) and parity ($P = 0.59$) did not affect pregnancy

loss [R32 = 12.2% (5/41) vs. SR = 8.5% (6/71); primiparous = 12.2% (5/41) vs. multiparous = 8.5% (6/71)].

Ovarian Structures and Progesterone Concentration at Nonpregnancy Diagnosis

Among cows with a CL ≥15 mm, 84.4% had P4 ≥1 ng/mL, 3.9% had P4 >0.5 and <1 ng/mL, and 11.7% had P4 ≤0.5 ng/mL. Among cows with no CL ≥15 mm at NPD, 29.5% had P4 ≥1 ng/mL, 5.1% had P4 >0.5 and < 1 ng/mL, and 65.4% had P4 ≤0.5 ng/mL. Among cows with no CL ≥15 mm and P4 ≥1 ng/mL at NPD, 43.5% (10/23) had at least one fluid filled cavity ≥25 mm (usually considered an ovarian cyst) present on their ovaries. The agreement between TUS and P4 concentration to determine the presence of a functional CL (P4 ≥1 ng/mL) was moderate [kappa = 0.53 (95% CI: 0.42 to 0.64); $P < 0.001$].

Ovarian Structures and Progesterone Concentration at the GnRH Treatment before Timed Artificial Insemination

Overall, a greater ($P = 0.02$) proportion of cows had P4 concentration <0.5 ng/mL in the SR (81.8%; 99/121) than the R32 treatment (68.4%; 78/114). In addition, for cows with a CL at NPD the proportion of cows with P4 ≤0.5 ng/mL tended ($P = 0.08$) to be greater for the SR than for the R32 treatment (Table 4.2). There was no effect of treatment ($P = 0.14$) for cows without a CL at NPD (Table 4.2). When the cutoff for low circulating P4 concentration was 1 ng/mL, the overall proportion of cows with P4 concentration below the cut off was greater ($P = 0.05$) for the SR (88.4%; 107/121) than for the R32 (79.0%; 90/114) treatment. The proportion of cows with P4 <1ng/mL was not affected by treatment ($P = 0.27$) among cows with a CL at NPD and tended to be affected ($P = 0.09$) by treatment among cows with no CL at NPD (Table 4.2).

Cows in the R32 treatment tended ($P = 0.10$) to have larger follicles (18.0 ± 0.4 mm) than cows in the SR treatment (17.1 ± 0.4 mm). For cows with a CL at NPD treatment did not affect ($P = 0.74$) size of the largest follicle at the time of GnRH (Table 4.2), whereas for cows with no CL at NPD follicle size at GnRH treatment tended ($P = 0.07$) to be greater for cows in the R32 treatment (Table 4.2).

Table 4.1. Pregnancy per AI and pregnancy loss for cows that received the experimental treatments.

Item	Treatment[1]		*P*-value
	R32 % (n/n)	SR % (n/n)	
Overall P/AI[2] 32 ± 3 d after AI for all inseminations	29.6 (182/615)	33.0 (247/748)	0.14
P/AI for CL cows[3]	31.9 (140/439)	31.6 (172/545)	0.94
P/AI for No CL cows[4]	23.9 (42/176)	37.0 (75/203)	< 0.01
Overall P/AI 63 ± 3 d after AI for all inseminations	27.0 (165/611)	28.0 (204/730)	0.62
Overall pregnancy loss for all inseminations	7.3 (13/178)	10.9 (25/229)	0.17

[1]Treatment = cows were randomly assigned to the D32-Resynch (R32) or Short Resynchronization (SR) treatments. Nonpregnancy diagnosis was performed 32 ± 3 d after AI. Nonpregnant cows in the R32 treatment received the Ovsynch protocol (GnRH-7 d-PGF2α-56 h-GnRH-16 to 18 h-TAI) for resynchronization of ovulation, whereas cows in the SR treatment were resynchronized based on the ovarian structures at nonpregnancy diagnosis. Cows with at least one corpus luteum ≥ 15 mm and a follicle ≥ 10 mm received PGF2α, 24 h later PGF2α, 32 h later GnRH, and TAI 16 to 18 h after GnRH, whereas cows that did not fulfill the CL and follicle criteria received a modified Ovsynch protocol with two PGF2α treatments and progesterone supplementation (GnRH plus CIDR-7 d-PGF2α plus CIDR removal-24 h- PGF2α-32 h-GnRH-16 to 18 h-TAI).

[2]P/AI = pregnancy per AI.

[3]Cows with at least one corpus luteum ≥ 15 mm and a follicle ≥ 10 mm

[4] Cows with no corpus luteum ≥ 15 mm and/or no follicle ≥ 10 mm

Table 4.2 Ovarian status at the time of GnRH treatment before timed AI for cows that received the experimental treatments.

	Group based on ovarian structures present at NPD[1]					
	CL[2] ≥ 15 mm and follicle ≥ 10 mm			No CL ≥ 15 mm and/or no follicle ≥ 10 mm		
Item	R32 (n = 80)	SR (n = 88)	*P*-value	R32 (n = 34)	SR (n = 33)	*P*-value
P4[1] ≤ 0.5 ng/mL (%; n)	70.0 (56)	81.8 (72)	0.08	64.7 (22)	81.8 (27)	0.14
P4 < 1 ng/mL (%; n)	81.3 (65)	87.5 (77)	0.27	73.5 (25)	90.9 (30)	0.09
Size of the largest follicle (mm)	17.1 ± 0.5	16.8 ± 0.4	0.74	19.9 ± 0.8	17.8 ± 0.9	0.07

[1]Treatment = cows were randomly assigned to the D32-Resynch (R32) or Short Resynchronization (SR) treatments. Nonpregnancy diagnosis was performed 32 ± 3 d after AI. Nonpregnant cows in the R32 treatment received the Ovsynch protocol (GnRH-7 d-PGF2α-56 h-GnRH-16 to 18 h-TAI) for resynchronization of ovulation, whereas cows in the SR treatment were resynchronized based on the ovarian structures at nonpregnancy diagnosis. Cows with at least one corpus luteum ≥ 15 mm and a follicle ≥ 10 mm received PGF2α, 24 h later PGF2α, 32 h later GnRH, and TAI 16 to 18 h after GnRH, whereas cows that did not fulfill the CL and follicle criteria received a modified Ovsynch protocol with two PGF2α treatments and progesterone supplementation (GnRH plus CIDR-7 d-PGF2α plus CIDR removal-24 h- PGF2α-32 h-GnRH-16 to 18 h-TAI).

[1]NPD = nonpregnancy diagnosis

[2]CL = corpus luteum

[3]P4 = progesterone

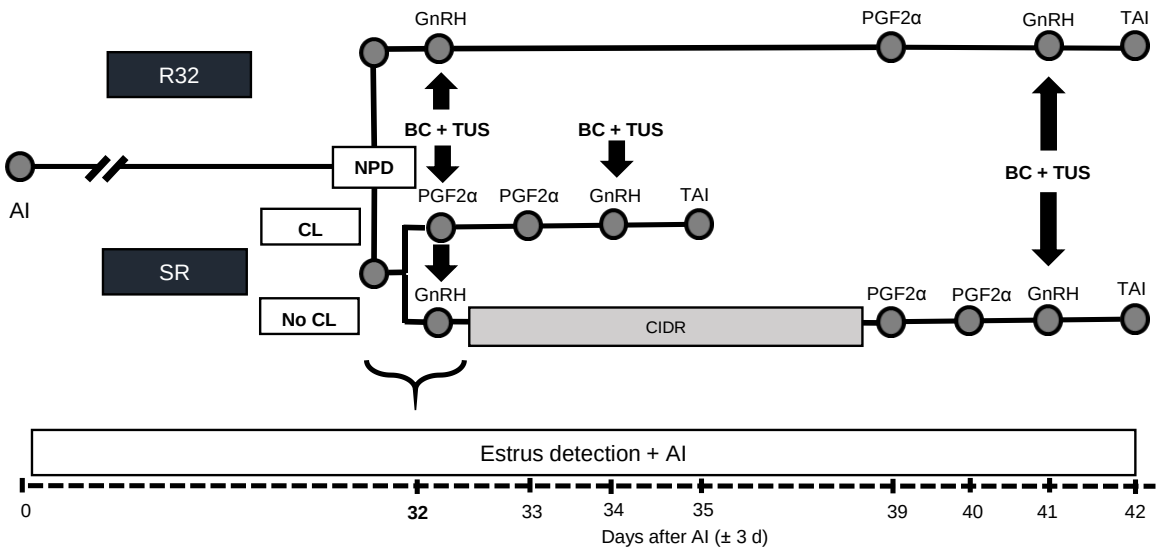

Figure 4.1. Graphical representation of experimental procedures. After first service postpartum cows were stratified by parity (primiparous vs. multiparous) and randomly assigned to the D32-Resynch (R32; n = 1,010) or Short Resynchronization (SR; n= 1,000) treatment. Nonpregnancy diagnosis (NPD) was performed 32 ± 3 d after AI, nonpregnant cows from the R32 treatment received the Ovsynch protocol (GnRH-7 d-PGF2α-56 h-GnRH-16 to 18 h-TAI) whereas cows in the SR treatment were resynchronized based on the ovarian structures present at nonpregnancy diagnosis. Cows with at least one corpus luteum ≥ 15 mm and follicle ≥ 10 mm received PGF2α, 24 h later PGF2α, 32 h later GnRH, and TAI 16 to 18 h after GnRH. Cows that did not fulfill these criteria received a modified Ovsynch protocol with two PGF2α treatments and progesterone (P4) supplementation (GnRH + CIDR-7 d-CIDR removal + PGF2α-24 h-PGF2α-32 h -GnRH-16 to 18 h-TAI). In a sub-group of cows blood samples were collected to measure P4 concentration at NPD and at the time of GnRH treatment before TAI. Size of the largest ovarian follicle was also estimated and recorded.
NPD = nonpregnancy diagnosis, CL = corpus luteum, TUS = transrectal ultrasonography, BC = blood collection, TAI = timed AI.

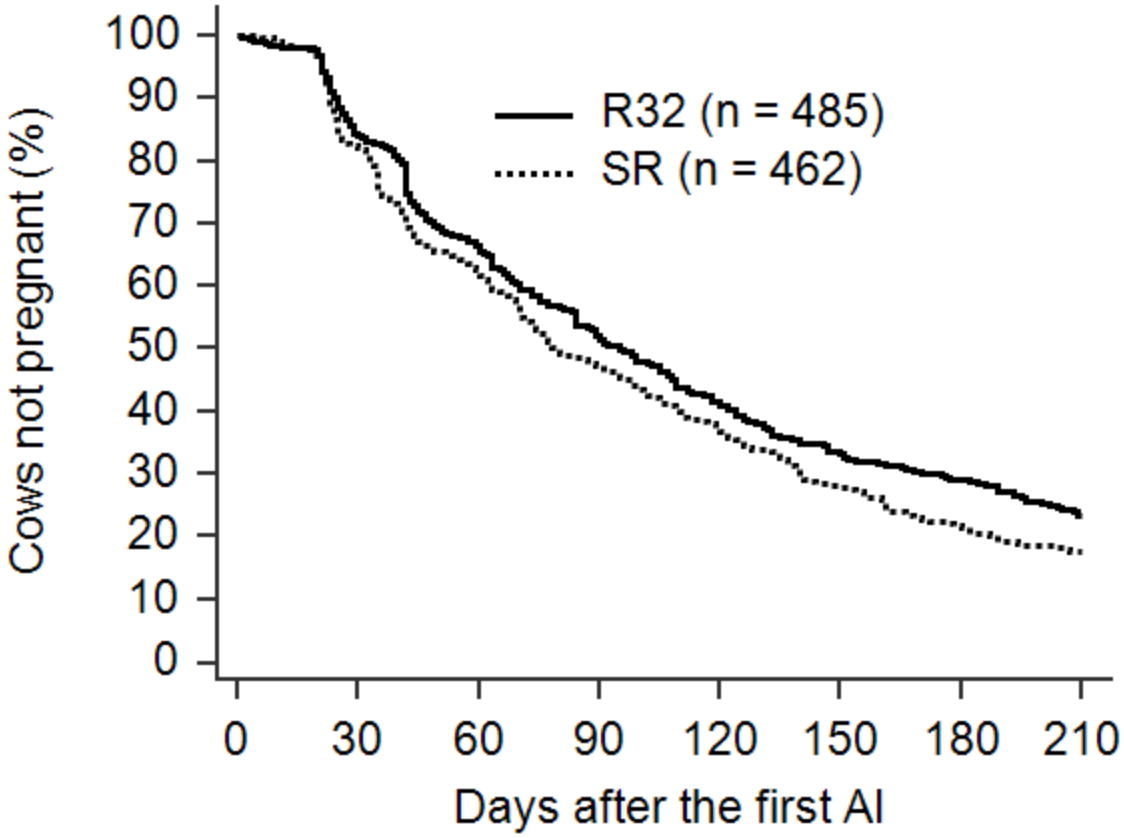

Figure 4.2. Kaplan-Meier survival curves for time to pregnancy from enrolment up to 210 d after the first service postpartum for cows in the D32-Resynch (R32) and Short Resynchronization (SR) treatments that failed to conceive after the first service. The hazard of pregnancy was affected (P = 0.03) by treatment (hazard ratio = 1.18, 95% CI: 1.01 to 1.37). Median time to pregnancy was 95 (95% CI: 84 to 108) and 79 d (95% CI: 71 to 96) for the R32 and SR treatments, respectively. Mean time to pregnancy was 111 ± 3 and 100 ± 3 d for the R32 and SR treatments, respectively.

DISCUSSION

The goal of reproductive management programs for second and greater AI services is to minimize the interbreeding interval and maximize conception risk for nonpregnant cows which, in turn minimizes time to pregnancy after the first service and reduces the proportion of cows leaving the herd due to reproductive failure. In our current experiment, a program that relied on resynchronization of ovulation treatments given to cows based on the ovarian structures present at NPD was compared with blanket use (i.e., regardless of ovarian structures present) of the D32-Resynch protocol. In support of our hypothesis, cows in the Short-Resynch (SR) program had fewer days to pregnancy after the first service. Another benefit of the SR treatment was a reduction in the proportion of nonpregnant cows at the end of experimental period (i.e., 210 d after first service corresponded with at ~250 to 295 DIM depending on DIM at first service). Thus, the SR treatment may benefit dairy herds when compared with blanket use of the D32-Resynch protocol through a reduction in both time to pregnancy during lactation and the proportion of nonpregnant cows removed from the herd at the end of lactation. Of note, the reduction in interbreeding interval for cows with a CL at NPD in the SR treatment was possible without the GnRH 25 ± 3 d after AI eliminating two unintended consequences: (1) interfering with estrus expression before NPD and (2) unnecessarily treating all pregnant cows with GnRH. In addition, we observed that cows with a CL ≥15 mm and a follicle ≥10 mm at NPD that received the short resynchronization protocol (PGF-24 h-PGF-32 h-GnRH-16 to 18 h -TAI) had similar P/AI than cows with the same ovarian status resynchronized with the D32-Resynch protocol. Conversely, cows that did not have a CL ≥15 mm and/or a follicle ≥10 mm at NPD clearly benefited from P4 supplementation and the two PGF2α treatments as they had greater P/AI than cows that received the D32-Resynch protocol.

Various strategies for resynchronization of ovulation to maximize P/AI (Dewey et al., 2010; Giordano et al., 2012b; Giordano et al., 2012c), shorten the interval between TAI services (Fricke et al., 2003; Galvão et al., 2007; Sinedino et al., 2014), or maximize the number of cows inseminated upon estrus detection (Bruno et al., 2013; Chebel et al., 2013; Giordano et al., 2015) have been developed and tested. Nonetheless, few experiments evaluated the effect of these strategies on time to pregnancy during lactation or after the first service, which is the ultimate driver of the positive relationship between good reproductive efficiency and dairy cow profitability. This is particularly relevant when cows are re-inseminated at different intervals because differences in interbreeding interval or re-insemination dynamics (i.e., proportion of cows AI at detected estrus vs. TAI) can compensate for a lack of gain or even reductions in P/AI in the group with shorter interbreeding interval. Indeed, the greater hazard of pregnancy for cows in the SR treatment in our experiment was primarily due to the 7 d shorter interbreeding interval for the ~70% of cows with a CL at NPD in spite of no gain in P/AI. The 13-percentage point greater P/AI without extending the interbreeding interval in cows with no CL at NPD in the SR treatment also contributed to reduce time to pregnancy; however, only ~30% of the total nonpregnant cows benefited from greater P/AI.

The similar P/AI for cows with CL in the SR than the D32-Resynch treatment was critical for reducing time to pregnancy because if cows that received the short resynchronization protocol would have had reduced P/AI, the benefit of shorter interbreeding interval could have been counterbalanced by the reduction in P/AI. For cows that did not receive GnRH to induce a new follicular wave in the SR treatment the detriment of suboptimal control of the follicular wave dynamics (Wijma et al., 2017) must have been compensated, at least in part, by the two PGF2α treatments to induce luteal regression. Indeed, the proportion of cows with a CL at NPD

that had low P4 concentration (for the 0.5 ng/mL cutoff) at the time of GnRH treatment before TAI tended to be greater in the SR treatment. We speculate that maximizing the proportion of cows with low circulating P4 concentration (i.e., ≤ 0.5 ng/mL) at the time of inducing ovulation before TAI had a greater effect on P/AI than reduced control of the follicular wave from which the ovulatory follicle emerged. In this regard, previous experiments with resynchronized lactating dairy cows reported greater P/AI for cows that failed to ovulate to the first GnRH treatment of Ovsynch but had complete luteolysis before TAI than for cows that ovulated in response to the GnRH treatment but had incomplete luteolysis before TAI (Giordano et al., 2012c). Other potential issues with synchronization of ovulation protocols that do not tightly control follicular wave dynamics before TAI include lack of ovulation to the GnRH before TAI and the potential to increase pregnancy losses due to poor oocyte quality or luteal insufficiency after insemination. Cows in which the follicular wave of the ovulatory follicle was initiated less than 5 d before induction of luteal regression may have either failed to ovulate in response to the GnRH before TAI (Vasconcelos et al., 1999) or ovulated a small follicle that led to the formation of a small CL incapable of supporting pregnancy (Vasconcelos et al., 2001). Cows that initiated the follicular wave of the ovulatory follicle more than 5 d before induction of luteal regression may have been more likely to have poor embryo quality because of ovulation of an aged oocyte around insemination (Cerri et al., 2009). All these issues were expected for some cows with CL at NPD in the SR treatment because GnRH was not given to induce a follicular wave from which the ovulatory follicle would emerge. In this regard, we recently reported greater variation for age of the largest follicle and greater follicle size at NPD 32 ± 3 d after AI in cows that did not receive GnRH compared with cows that received GnRH 7 d earlier (Wijma et al., 2017). Although our observations need confirmation with additional data, we speculate that the

tendency for greater pregnancy loss in the SR treatment for CL cows could be explained, at least in part, by reduced control of the follicular wave dynamics before TAI. In our experiment; however, the differences in pregnancy loss between groups may have also been less dramatic because the follicular wave dynamics of cows resynchronized with Ovsynch-type protocols is not optimal due to the high proportion of previously inseminated cows that fail to ovulate in response to the first GnRH of the protocol (Giordano et al., 2012c; Lopes et al., 2013; Wijma et al., 2017). In particular, poor ovulatory response has been reported for cows with a functional CL at the time of the GnRH treatment (Galvão and Santos, 2010; Giordano et al., 2012c; Lopes et al., 2013).

In spite of no reduction of the interbreeding interval for No CL cows, the substantial increment in P/AI from 23.9 to 37.0% likely contributed to the overall reduction of time to pregnancy for the SR treatment. Greater P/AI for resynchronized inseminations that occur at the same interbreeding interval reduces overall time to pregnancy by decreasing the need for re-inseminations at a later time either at detected estrus or TAI. In cows with No CL at NPD, the beneficial effect of P4 supplementation and two PGF2α treatments likely synergized. Supplemental P4 in cows without a CL at the initiation of Ovsynch-type protocols has been proven effective to increase P/AI (Bartolome et al., 2009; Bisinotto et al., 2013; Bisinotto et al., 2015) due to a combination of physiological mechanisms including: (1) improved embryo quality because of improved endocrine environment for oocyte development (Rivera et al., 2011), (2) improved uterine environment (Cerri et al., 2011), and (3) reduction of premature estrus and ovulation before TAI (Stevenson et al., 2006). The latter, however, may not have been as relevant in the current experiment because cows detected in estrus any time were immediately inseminated and had reasonable P/AI. An additional PGF2α treatment after induction of luteal

regression is known to increase the proportion of cows with complete luteolysis before TAI (Brusveen et al., 2009; Ribeiro et al., 2012; Wiltbank et al., 2015) which, in turn, increases P/AI after TAI services (Souza et al., 2007; Brusveen et al., 2009; Santos et al., 2016). In our experiment, the additional PGF2α treatment may explain a substantial portion of the gain in P/AI for cows with No CL at NPD. Cows without a functional CL at the beginning of Ovsynch-type protocols are more likely to ovulate after GnRH (Galvão and Santos, 2010; Giordano et al., 2012c; Lopes et al., 2013) due to a greater GnRH-induced LH response (Giordano et al., 2012a; Lima et al., 2013; Pulley et al., 2015) thereby, are more likely to have only one 6 d old CL not fully responsive to PGF2α at the time of induction of luteolysis. That may explain the relatively low proportion of cows with low P4 at the time of the GnRH before TAI even for cows that received two PGF2α treatments. Our results should be interpreted with caution because they do not necessarily represent actual luteal regression as we did not have data for P4 concentration at the time of the PGF2α treatment in these cows.

Accurate determination of the physiological status of cows through identification of ovarian structures is important for the success of reproductive management strategies relying on treatments designed for a particular ovarian status. In the current experiment, the proportion of nonpregnant cows bearing a CL 32 ± 3 observed was in agreement with previous research (Bruno et al., 2014; Giordano et al., 2015; Wijma et al., 2017). Nevertheless, the agreement between TUS and P4 concentration for the determination of the presence of an active CL was only moderate and lower than that reported in other experiments from our laboratory (Giordano et al., 2015) and others (Bicalho et al., 2008). Lack of agreement between TUS and P4 was expected for some cows because of the limitations of B-mode ultrasonography to predict the P4 secretion ability of luteal tissue (Battocchio et al., 1999), lack of a strong linear correlation

between CL size and P4 secretion (Sprecher et al., 1989; Mann, 2009), and the ability of fluid-filled cavities with apparently no luteal tissue or a small amount of luteal tissue to produce P4 (Farin et al., 1990; Giordano et al., 2016). For example, the 11.4% of cows with P4 <0.5 ng/mL and a CL ≥15 mm visualized through TUS may have had a CL that completed functional but not structural luteolysis, whereas the 3.9% of cows with P4 ≥0.5 and <1 ng/mL were likely to have a CL from a recent ovulation. Applying the short resynchronization of ovulation treatment in these cows may have been detrimental because cows with an already regressed CL may have shown estrus prematurely, whereas incomplete luteolysis could have been limiting in cows with an immature CL at the time of induction of luteolysis (Henricks et al., 1974; Wiltbank et al., 2015). Although not evaluated in this experiment, we speculate that these two subgroups of cows may have had better performance if they would have received the modified Ovsynch protocol with P4 supplementation. In particular, the group with an immature CL because more time until induction of luteolysis would have allowed the CL to become fully responsive to PGF2α. On the other hand, almost half (i.e., 43.5%) of the cows with no CL ≥15 mm visualized through TUS and P4 ≥1 ng/mL had a fluid filled cavity ≥25 mm (usually considered ovarian cysts) with the potential to produce P4. The rest of cows with P4 >0.5 ng/mL may have had a small CL formed after a recent ovulation but not easily visualized through TUS or a CL <15 mm in diameter. Unfortunately, it is not possible to determine if cows with a small but mature CL visualized through TUS would have benefited more by the 7 d shorter interbreeding interval if they received the short resynchronization protocol than by receiving the treatment for cows with no CL. Conversely, the other subgroups of cows likely benefited by receiving the modified Ovsynch protocol with P4 supplementation. Until more accurate methods to determine circulating concentration of P4 become available for on-farm use, a small proportion of cows will be

misclassified based on TUS into the CL or no CL group and receive a treatment that may not necessarily optimize their fertility or justify delaying their re-insemination.

Although the control group in this experiment could have easily incorporated the modified protocol with two PGF2α treatments and P4 supplementation for cows with no CL at NPD, our reasoning to not do so was to maintain the treatment as simple as possible to reflect the conditions of many dairy herds in the U.S. that use this strategy for resynchronization of ovulation (Caraviello et al., 2006; Ferguson and Skidmore, 2013; Scott, 2016). Most farms likely choose to either not treat cows based on their ovarian status at NPD 32 ± 3 d after AI or even conduct NPD 39 ± 3 d coincident with the time of the PGF2α treatment before TAI. Whether differential treatment of cows with no CL at NPD 32 ± 3 d after AI in the R32 treatment would have offset the gain in reproductive performance from the 7 d shorter interbreeding interval in the SR treatment is unknown. Nevertheless, it is rather unlikely because the majority of nonpregnant cows 32 ± 3 d after AI had a CL at NPD thereby, they benefited by the reduced interbreeding interval of the SR protocol.

Finally, another important consideration for the use of the SR treatment as a management strategy in dairy farms is the potential extra cost of the program as compared with blanket use of D32-Resynch. The expected reduction in time to pregnancy and proportion of cows not pregnant in late lactation for the SR treatment must offset the additional cost of the synchronization protocol for cows without a CL at NPD and the potential additional cost of conducting TUS in farms that do not routinely use TUS for NPD. Thus, the economics of more complex and potentially more expensive management strategies such as SR warrants further investigation.

CONCLUSIONS

A reproductive management program designed to (1) reduce the interbreeding interval for TAI services in cows with a CL ≥15 mm and a follicle ≥10 mm at NPD and (2) increase P/AI of cows without a CL at NPD through a modified Ovsynch protocol with two PGF2α treatments and P4 supplementation, reduced time to pregnancy during lactation and the proportion of nonpregnant cows 210 d after first service when compared with blanket use of the D32-Resynch protocol. Therefore, the SR program is a new management strategy for second and greater AI services with the potential to improve reproductive performance of dairy herds that enroll all nonpregnant cows not re-inseminated at detected estrus in typical Ovsynch-type resynchronization of ovulation protocols regardless of their ovarian status. Removing the GnRH treatment to induce a new follicular wave in the SR treatment not only helped reduce the interbreeding interval for a majority of cows without disrupting estrous behavior but also reduced unnecessary treatment of pregnant cows with GnRH 25 ± 3 d after their previous insemination.

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REFERENCES

Abramson, J. H. 2011. WINPEPI updated: computer programs for epidemiologists, and their teaching potential. Epidemiologic Perspectives & Innovations 8:1.

Bartolome, J., F. Silvestre, S. Kamimura, A. Arteche, P. Melendez, D. Kelbert, J. McHale, K. Swift, L. Archbald, and W. Thatcher. 2005. Resynchronization of ovulation and timed insemination in lactating dairy cows: I: use of the Ovsynch and Heatsynch protocols after non-pregnancy diagnosis by ultrasonography. Theriogenology 63:1617-1627.

Bartolome, J., J. Van Leeuwen, M. Thieme, O. Sa'Filho, P. Melendez, L. Archbald, and W. Thatcher. 2009. Synchronization and resynchronization of inseminations in lactating dairy cows with the CIDR insert and the Ovsynch protocol. Theriogenology 72:869-878.

Battocchio, M., G. Gabai, A. Mollo, M. Veronesi, F. Soldano, G. Bono, and F. Cairoli. 1999. Agreement between ultrasonographic classification of the CL and plasma progesterone concentration in dairy cows. Theriogenology 51:1059-1069.

Berndtson, A., S. Vincent, and J. Fortune. 1995. Low and high concentrations of gonadotropins differentially regulate hormone production by theca interna and granulosa cells from bovine preovulatory follicles. Biology of reproduction 52:1334-1342.

Bicalho, R., K. Galvao, C. Guard, and J. Santos. 2008. Optimizing the accuracy of detecting a functional corpus luteum in dairy cows. Theriogenology 70:199-207.

Bisinotto, R., L. Castro, M. Pansani, C. Narciso, N. Martinez, L. Sinedino, T. Pinto, N. Van de Burgwal, H. Bosman, and R. Surjus. 2015. Progesterone supplementation to lactating dairy cows without a corpus luteum at initiation of the Ovsynch protocol. Journal of dairy science 98:2515-2528.

Bisinotto, R., E. Ribeiro, F. Lima, N. Martinez, L. Greco, L. Barbosa, P. Bueno, L. Scagion, W. Thatcher, and J. Santos. 2013. Targeted progesterone supplementation improves fertility in lactating dairy cows without a corpus luteum at the initiation of the timed artificial insemination protocol. Journal of dairy science 96:2214-2225.

Bruno, R., A. Farias, J. Hernández-Rivera, A. Navarrette, D. Hawkins, and T. Bilby. 2013. Effect of gonadotropin-releasing hormone or prostaglandin F 2α-based estrus synchronization programs for first or subsequent artificial insemination in lactating dairy cows. Journal of dairy science 96:1556-1567.

Bruno, R., J. Moraes, J. Hernández-Rivera, K. Lager, P. Silva, A. Scanavez, L. Mendonça, R. Chebel, and T. Bilby. 2014. Effect of an Ovsynch56 protocol initiated at different intervals after insemination with or without a presynchronizing injection of gonadotropin-releasing hormone on fertility in lactating dairy cows. Journal of dairy science 97:185-194.

Brusveen, D., A. Souza, and M. Wiltbank. 2009. Effects of additional prostaglandin F 2α and estradiol-17β during Ovsynch in lactating dairy cows. Journal of dairy science 92:1412-1422.

Caraviello, D., K. Weigel, P. Fricke, M. Wiltbank, M. Florent, N. Cook, K. Nordlund, N. Zwald, and C. Rawson. 2006. Survey of management practices on reproductive performance of dairy cattle on large US commercial farms. Journal of dairy science 89:4723-4735.

Cerri, R., R. Chebel, F. Rivera, C. Narciso, R. Oliveira, M. Amstalden, G. Baez-Sandoval, L. Oliveira, W. Thatcher, and J. Santos. 2011. Concentration of progesterone during the development of the ovulatory follicle: II. Ovarian and uterine responses. Journal of dairy science 94:3352-3365.

Cerri, R. L., H. M. Rutigliano, R. C. Chebel, and J. E. Santos. 2009. Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. Reproduction 137:813-823.

Chebel, R., A. Scanavez, P. Silva, J. Moraes, L. Mendonça, and G. Lopes. 2013. Evaluation of presynchronized resynchronization protocols for lactating dairy cows. Journal of dairy science 96:1009-1020.

Dewey, S., L. Mendonça, G. Lopes, F. Rivera, F. Guagnini, R. Chebel, and T. Bilby. 2010. Resynchronization strategies to improve fertility in lactating dairy cows utilizing a presynchronization injection of GnRH or supplemental progesterone: I. Pregnancy rates and ovarian responses. Journal of dairy science 93:4086-4095.

Farin, P., R. Youngquist, J. Parfet, and H. Garverick. 1990. Diagnosis of luteal and follicular ovarian cysts in dairy cows by sector scan ultrasonography. Theriogenology 34:633-642.

Ferguson, J. D. and A. Skidmore. 2013. Reproductive performance in a select sample of dairy herds. Journal of dairy science 96:1269-1289.

Fricke, P., D. Caraviello, K. Weigel, and M. Welle. 2003. Fertility of dairy cows after resynchronization of ovulation at three intervals following first timed insemination. Journal of dairy science 86:3941-3950.

Galvão, K. and J. Santos. 2010. Factors affecting synchronization and conception rate after the Ovsynch protocol in lactating Holstein cows. Reproduction in domestic animals 45:439-446.

Galvão, K., J. Santos, R. Cerri, R. Chebel, H. Rutigliano, R. Bruno, and R. Bicalho. 2007. Evaluation of methods of resynchronization for insemination in cows of unknown pregnancy status. Journal of dairy science 90:4240-4252.

Giordano, J., P. Fricke, J. Guenther, G. Lopes, M. Herlihy, A. Nascimento, and M. Wiltbank. 2012a. Effect of progesterone on magnitude of the luteinizing hormone surge induced by two different doses of gonadotropin-releasing hormone in lactating dairy cows. Journal of dairy science 95:3781-3793.

Giordano, J., M. Stangaferro, R. Wijma, W. Chandler, and R. Watters. 2015. Reproductive performance of dairy cows managed with a program aimed at increasing insemination of cows in estrus based on increased physical activity and fertility of timed artificial inseminations. Journal of dairy science 98:2488-2501.

Giordano, J., M. Thomas, G. Catucuamba, M. Curler, M. Masello, M. Stangaferro, and R. Wijma. 2016. Reproductive management strategies to improve the fertility of cows with a suboptimal response to resynchronization of ovulation. Journal of dairy science 99:2967-2978.

Giordano, J., M. Wiltbank, J. Guenther, M. Ares, G. Lopes, M. Herlihy, and P. Fricke. 2012b. Effect of presynchronization with human chorionic gonadotropin or gonadotropin-releasing hormone 7 days before resynchronization of ovulation on fertility in lactating dairy cows. Journal of dairy science 95:5612-5625.

Giordano, J., M. Wiltbank, J. Guenther, R. Pawlisch, S. Bas, A. Cunha, and P. Fricke. 2012c. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. Journal of dairy science 95:639-653.

Henricks, D., J. Long, J. Hill, and J. Dickey. 1974. The effect of prostaglandin F2α during various stages of the oestrous cycle of beef heifers. Journal of reproduction and fertility 41:113-120.

Jo, M. and J. Fortune. 2003. Changes in oxytocin receptor in bovine preovulatory follicles between the gonadotropin surge and ovulation. Molecular and cellular endocrinology 200:31-43.

Komar, C., A. Berndtson, A. Evans, and J. Fortune. 2001. Decline in Circulating Estradiol During the Periovulatory Period Is Correlated with Decreases in Estradiol and Androgen, and in

Messenger RNA for P450 Aromatase and P450 17α-Hydroxylase, in Bovine Preovulatory Follicles 1. Biology of reproduction 64:1797-1805.

Lima, F., E. Ribeiro, R. Bisinotto, L. Greco, N. Martinez, M. Amstalden, W. Thatcher, and J. Santos. 2013. Hormonal manipulations in the 5-day timed artificial insemination protocol to optimize estrous cycle synchrony and fertility in dairy heifers. Journal of dairy science 96:7054-7065.

Lopes, G., J. Giordano, A. Valenza, M. Herlihy, J. Guenther, M. Wiltbank, and P. Fricke. 2013. Effect of timing of initiation of resynchronization and presynchronization with gonadotropin-releasing hormone on fertility of resynchronized inseminations in lactating dairy cows. Journal of dairy science 96:3788-3798.

Louca, A. and J. Legates. 1968. Production Losses in Dairy Cattle Due to Days Open. Journal of Dairy Science 51:573-583.

Mann, G. 2009. Corpus luteum size and plasma progesterone concentration in cows. Animal reproduction science 115:296-299.

Mendonça, L. G., S. T. Dewey, G. Lopes, F. A. Rivera, F. S. Guagnini, J. P. Fetrow, T. R. Bilby, and R. C. Chebel. 2012. Effects of resynchronization strategies for lactating Holstein cows on pattern of reinsemination, fertility, and economic outcome. Theriogenology 77:1151-1158.

Moreira, F., C. Orlandi, C. Risco, R. Mattos, F. Lopes, and W. Thatcher. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. Journal of Dairy Science 84:1646-1659.

Oltenacu, P., T. Rounsaville, R. Milligan, and R. Foote. 1981. Systems analysis for designing reproductive management programs to increase production and profit in dairy herds. journal of Dairy Science 64:2096-2104.

Pulley, S. L., D. Keisler, and J. Stevenson. 2015. Concentrations of luteinizing hormone and ovulatory responses in dairy cows before timed artificial insemination. Journal of dairy science 98:6188-6201.

Pursley, J., M. Mee, and M. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF 2α and GnRH. Theriogenology 44:915-923.

Remnant, J., M. Green, J. Huxley, and C. Hudson. 2015. Variation in the interservice intervals of dairy cows in the United Kingdom. Journal of dairy science 98:889-897.

Ribeiro, E., R. Bisinotto, M. Favoreto, L. Martins, R. Cerri, F. Silvestre, L. Greco, W. Thatcher, and J. Santos. 2012. Fertility in dairy cows following presynchronization and administering twice the luteolytic dose of prostaglandin F 2α as one or two injections in the 5-day timed artificial insemination protocol. Theriogenology 78:273-284.

Rivera, F. A., L. G. Mendonça, G. Lopes, J. E. Santos, R. V. Perez, M. Amstalden, A. Correa-Calderón, and R. C. Chebel. 2011. Reduced progesterone concentration during growth of the first follicular wave affects embryo quality but has no effect on embryo survival post transfer in lactating dairy cows. Reproduction 141:333-342.

Santos, V., P. Carvalho, C. Maia, B. Carneiro, A. Valenza, P. Crump, and P. Fricke. 2016. Adding a second prostaglandin F 2α treatment to but not reducing the duration of a PRID-Synch protocol increases fertility after resynchronization of ovulation in lactating Holstein cows. Journal of dairy science 99:3869-3879.

Scott, B. D. 2016. Incorporation of dairy farm survey data and epidemiological patterns for agent-based simulation modeling of dairy herd dynamics. MS Thesis. Cornell University.

Sinedino, L., F. Lima, R. Bisinotto, R. Cerri, and J. Santos. 2014. Effect of early or late resynchronization based on different methods of pregnancy diagnosis on reproductive performance of dairy cows. Journal of dairy science 97:4932-4941.

Souza, A., H. Ayres, R. Ferreira, and M. Wiltbank. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology 70:208-215.

Souza, A., A. Gümen, E. Silva, A. Cunha, J. Guenther, C. Peto, D. Caraviello, and M. Wiltbank. 2007. Supplementation with estradiol-17β before the last gonadotropin-releasing hormone injection of the Ovsynch protocol in lactating dairy cows. Journal of dairy science 90:4623-4634.

Sprecher, D., R. Nebel, and S. Whitman. 1989. The predictive value, sensitivity and specificity of palpation per rectum and transrectal ultrasonography for the determination of bovine luteal status. Theriogenology 31:1165-1172.

Stevenson, J., J. Pursley, H. Garverick, P. Fricke, D. Kesler, J. Ottobre, and M. Wiltbank. 2006. Treatment of cycling and noncycling lactating dairy cows with progesterone during Ovsynch. Journal of dairy science 89:2567-2578.

Vasconcelos, J., R. Sartori, H. Oliveira, J. Guenther, and M. Wiltbank. 2001. Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. Theriogenology 56:307-314.

Vasconcelos, J., R. Silcox, G. Rosa, J. Pursley, and M. Wiltbank. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology 52:1067-1078.

Wijma, R., M. L. Stangaferro, M. Masello, G. E. Granados, and J. O. Giordano. 2017. Resynchronization of ovulation protocols for dairy cows including or not including gonadotropin-releasing hormone to induce a new follicular wave: Effects on re-insemination pattern, ovarian responses, and pregnancy outcomes. Journal of Dairy Science (In Press).

Wiltbank, M. C., G. M. Baez, F. Cochrane, R. V. Barletta, C. R. Trayford, and R. T. Joseph. 2015. Effect of a second treatment with prostaglandin F2α during the Ovsynch protocol on luteolysis and pregnancy in dairy cows. Journal of dairy science 98:8644-8654.

Wiltbank, M. C. and J. R. Pursley. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. Theriogenology 81:170-185.

SECTION II

THE VAGINA AS A ROUTE OF HORMONE DELIVERY FOR SYNCHRONIZATION OF OVULATION IN CATTLE

CHAPTER I

INTRAVAGINAL DELIVERY OF PROSTAGLANDIN F2α AND GONADOTROPIN RELEASING HORMONE

1. General introduction

Synchronization of estrus and/or ovulation programs have been widely adopted in both beef (Lamb et al., 2010) and dairy (Wiltbank and Pursley, 2014) cattle production systems. The development of these programs has allowed the dairy industry to reduce time to pregnancy through timely insemination after the end of the voluntary waiting period (Pursley et al., 1997) and between inseminations not resulting in pregnancy (Fricke et al., 2003; Giordano et al., 2012). Some of these programs not only ensure timely insemination but also lead to greater pregnancy risk than insemination after a detected estrus (Borchardt et al., 2017). Despite the widespread use of synchronization of ovulation programs, the need to treat females multiple times with different hormones before insemination continues to limit adoption by cattle operations and is a disadvantage for correct implementation. Lack of compliance with protocols is one of the main reasons for poor outcomes during synchronization of ovulation protocols (Stevenson and Phatak, 2005; Galvão and Santos, 2010; Macmillan, 2010), mainly because finding cows and performing treatments at specific time points requires adequate facilities and access to labor. Furthermore, the need to either restrain cows in head gates, sort in sorting pens, or force them to pass through

palpation rails multiple times during each round of synchronization disrupts cow time budgets and may limit the ability of cows to express other behaviors such as feeding and resting when desired.

Thus, automating hormone delivery for synchronization of ovulation may be an alternative to successfully overcome some of the main limitations of proper implementation of synchronization of ovulation protocols for TAI. For example, automating hormone delivery may help farms with limited access to qualified labor or inadequate facilities to apply treatments and may also improve cow performance and welfare through a reduction in cow manipulation. Treatment automation may be possible through an electronically controlled device loaded with the hormones to be administered to synchronize ovulation. The device could be placed in an easily accessible body cavity (e.g., vagina) to deliver the appropriate dose of hormones at pre-defined time points and be removed at the time of insemination. Therefore, the number of times that cows would have to be located and the numbers of handlings would decrease dramatically. Automating hormone delivery could also maximize protocol compliance through proper dosing and timing of treatments, allow to more closely mimic physiological hormone release patterns, and tailor hormonal treatments to individual cows based on their specific physiological status. Even though there has been some interest in the past to develop this kind of technology (Cross et al., 2004; Künnemeyer et al., 2004), published research regarding the effectiveness of the intravaginal (IVG) route for PGF2α and GnRH delivery is scarce. The limited research performed in cattle (Zdunczyk et al., 1994; Heinonen et al., 1996) and other species (Okada et al., 1982; Viudes-De-Castro et al., 2007; Stewart et al., 2010) has shown intriguing results and suggest that the vagina may be a feasible route of PGF2α and GnRH delivery. This chapter

intends to review relevant information regarding current knowledge of intravaginal delivery of PGF2α and GnRH in cattle and other animal species.

2. Anatomy, histology, and physiology of the vagina in bovine females

The vagina is a fibromuscular tubular organ located caudal from the cervix and cranial to the vestibule. In the adult cow the vagina measures approximately 25 cm (Blazquez et al., 1989). The vagina receives irrigation from the uterine artery and the vaginal artery, and it is drained by a rich venous plexus which returns through the vaginal vein and accessory vaginal vein to the internal iliac vein (Dyce et al., 2009).

The histology of the vaginal epithelium of the cow is heterogeneous across the different portions of the organ and through the different stages of the estrous cycle. Using vaginas obtained from slaughtered cows at different stages of the estrous cycle, Cole (1930) described in detail the cyclic changes experienced by the vaginal mucosa. During proestrus, the caudal part of the vagina, 6 to 10 cm from the cervix until the urethral opening, showed a two layer flat epithelium, and an edematous highly vascularized stroma. During estrus, the epithelium became stratified columnar and there was major lymphocytic infiltration with epithelial breakdown and blood extravasation. Ten to eleven days after estrus (i.e., diestrus phase), the vaginal epithelium was almost entirely disrupted and in some portions limited to the germinal layer. The portion of the vagina immediately caudal to the cervix (i.e., cranial portion) is the one that experiences the most dramatic changes during the estrous cycle. During proestrus, the mucosa is covered by a columnar epithelium composed by 2 to 8 layers of cells. The superficial layer contains large mucus secreting cells. During estrus, most of the extension of the epithelium is reduced to a single layer of very active mucus secreting cells, nevertheless the size of these cells provides the

epithelium with a considerable thickness. During diestrus, the epithelium consists of two to four layers composed by a mixture of flattened and columnar cells. Cole (1930) also described the presence of mucosal folds. This folds are longitudinal in the caudal portion and become more tortuous as they get closer to the cervix where depth also increases to up as much as 1 mm.

The vaginal cavity is bathed by cervical and vaginal mucus, which varies in quantity, viscosity, and pH across the estrous cycle. Schilling and Zust (1968) performed a study where vaginal pH was measured at different stages of the estrous cycle and at different portions of the vagina. They observed an increasing gradient with the lowest pH in the most cranial portion and the highest in the most caudal zone. Moreover, pH also changed among phases of the estrous cycle: it was more acidic during estrus (6.45 ± 0.24 in the most cranial portion) and closer to neutrality during diestrus (6.94 ± 0.28 in the most cranial portion). Furthermore, they observed changes in viscosity, which started to decrease during proestrus, reached its minimum at the time of ovulation, and rapidly increased thereafter.

3. Intravaginal hormone delivery

Due to its ease of access, high permeability to low molecular weight molecules, and the presence of a rich venous and lymphatic drainage, the vagina is a promising alternative route for parenteral hormone delivery (Hussain and Ahsan, 2005). Further, the size and position of the vagina in cattle may allow to hold hormone delivery systems for prolonged periods of time.

A prerequisite for successful hormone delivery through body cavities is efficient absorption and absence of factors or molecules that cause degradation or modification of the hormone of interest before absorption in the body cavity. Hormone transport across membranes can occur in three different manners: a) transcellular transport by diffusion through cell walls due

to a concentration gradient, b) receptor mediated or vesicular transport, and c) intercellular transport by diffusion through intercellular tight junctions (Richardson and Illum, 1992). Absorption of substances through the vaginal mucosa is determined by factors inherent to the nature of the molecule, as well as anatomical and physiological factors. The latter refer mainly to the cyclic changes in epithelium structure and mucus abundance described above.

Physicochemical properties of hormones, including molecular weight, charge, lipophilicity, and ionization also affect absorption (Richardson and Illum, 1992; Hussain and Ahsan, 2005). Experiments in monkeys (Owada et al., 1977) and rabbits (Hwang et al., 1976) have shown that vaginal permeability to unbranched aliphatic alcohols increased with chain length and lipophilicity. Despite this, it is well accepted that lower molecular weight lipophilic hormones are better absorbed than larger molecules (Hussain and Ahsan, 2005). Moreover, Flynn et al. (1976) observed a greater permeability of the rabbit's vaginal mucosa to lipophilic steroids (e.g., progesterone, estrone) than to hydrophilic steroids (e.g., hydrocortisone, testosterone). Furthermore, due to the aqueous nature of the vaginal fluid, any molecule that is to be administered through the vaginal route must be soluble in water to some extent (Hussain and Ahsan, 2005).

In cattle, the intravaginal route has been used for more than 30 years primarily for P4 supplementation in synchronization of ovulation and/or estrus treatments (Webel, 1976; Macmillan et al., 1991; Macmillan and Peterson, 1993). Progesterone controlled release intravaginal devices consist of a silicone matrix containing a homogeneous dispersion of P4 allowing gradual release and absorption through the vaginal mucosa (Rathbone and Burke, 2013). This system has been widely adopted by farmers and veterinarians and is used in many synchronization of ovulation protocols for both dairy and beef cattle. On the other hand,

hormones such as PGF2α and GnRH that exert their effect in an acute manner need to be rapidly absorbed by the mucosa to reach their target tissues and cells to induce an acute response (Karsch et al., 1997; Niswender et al., 2000). Therefore, sustained release does not seem to be a feasible option for these reproductive hormones. Administration of GnRH and PGF2α through the intravaginal route requires a delivery system that can automatically control the release of the desired amount of hormone at once at predefined time points so that the physiological effect can be achieved (i.e., GnRH-induced LH surge for induction of ovulation and luteal regression for PGF2α). The device should allow programmed release of each hormone at a given time to eliminate the need for repeated treatments through injections and to mimic physiological patterns of secretion more closely so that the response to protocols can be maximized. In this regard, Cross et al. (2004) reported the design of a device that fulfils most of the requirements of an automated controlled device for synchronization of ovulation. It allowed to release a pre-programed or externally controlled amount of hormone into the vagina, thus enabling to perform treatments at different times without the need to interact with the cow. The device also had the ability to record body temperature and these data could be monitored in real time. In a publication from the same laboratory, Künnemeyer et al. (2004) described a similar device equipped with an antenna to remotely control timing and amount of hormone to be released. These devices could hold up to 40 mL, and were very accurate to deliver the desired amount of volume at the programed times (volume accuracy ~ 3%). Despite this, the fact that devices consisted of a single chamber would be one of the limitations for its use on synchronization of ovulation protocols that require treatment with different hormones.

Although the availability of multiple technologies for automated cow monitoring and management (Sørensen et al., 2016; Stangaferro et al., 2016) has increased, the refinement of

electronically controlled intravaginal hormone release devices available for commercial use, has not been explored extensively. Developing these devices would allow to reduce human interactions with cows as well as number of injections, improving cow welfare. Further, it would also allow to maximize treatment compliance and tailor protocols to the individual cow level. Hence, further research is required to develop devices capable of delivering multiple hormones at different times, and to test the feasibility of using the vagina as a route of administration for GnRH and PGF2α in cattle.

4. Intravaginal administration of Prostaglandin F2α

Prostaglandin F2α is an eicosanoid molecule synthetized in cattle by the endometrium among other tissues. It is derived from arachidonic acid which is released from cell membrane phospholipids by phospholipase A_2 and converted to prostaglandin H2 by cyclooxygenase and finally converted into PGF2α by PGF synthase (Funk, 2001). Like most eicosanoid molecules, PGF2α has a short half-life being totally metabolized in the lungs within a few (~ < 9) minutes (Shrestha et al., 2012). In cattle, PGF2α is responsible for triggering luteolysis and it has been used to synchronize estrus and ovulation for more than 30 years (Manns and Hafs, 1976; Pursley et al., 1995; Moreira et al., 2001). Luteolysis and its importance for synchronization of ovulation programs are discussed in detail in Chapter I of Section I.

The most commonly used PGF2α synthetic analogues are Dinoprost and Cloprostenol. The first one is structurally homologous to the natural form of PGF2α and therefore has a half-life of 7 to 8 minutes (Kimball et al., 1976). Conversely, due to the addition of an oxyaryl function to its molecule, Cloprostenol has a slower metabolization rate and a half-life of approximately 3 hours (Reeves, 1978; Bourne et al., 1980). Another difference between these

analogues is their potency. Whereas the luteolytic dose of Dinoprost in cattle is 25 mg, the dose of Cloprostenol to cause luteolysis is 0.5 mg (Dukes et al., 1974).

Information regarding IVG administration of PGF2α and its analogues in cattle is scarce. In an experiment using non-lactating *Bos indicus* cattle, Heinonen et al. (1996) tested the efficacy of IVG administration of Cloprostenol to induce estrus expression. Cows (n = 73) were either treated with 175 µg of Cloprostenol through the IVG route or with 500 µg of Cloprostenol intramuscular (i.m.). Estrus detection was performed for 12 days and cows not detected in estrus were treated again. Three days after the second treatment, cows that failed to express estrous behavior were examined by transrectal palpation and those presenting prominent uterine tone and/or vaginal discharge were considered to be in estrus. The experiment lacked a negative control and the statistical comparison was performed against an assumed 4.8% of daily estrus detection in non-treated cows. Estrus response was considered to be greater for cows that received IVG (62.5%) and i.m. (60.6%) treatment with Cloprostenol than for the modeled population. Although presence of a CL at the time of treatment and P4 concentration dynamics after treatment were not determined, the similar proportion of cows detected in estrus was similar for the groups treated with Cloprostenol through the IVG and i.m. route, suggested that IVG administration of PGF2α analogues might induce luteal regression in cattle.

In a more controlled experiment, Zdunczyk et al. (1994) observed an effect of IVG treatment with Cloprostenol on circulating P4 concentration. Cows (n = 22) presenting an active CL (P4 > 2 ng/mL in circulation) were treated with 500 µg of Cloprostenol either through the IVG or i.m route. Blood samples were collected at four hour intervals from 0 to 12 hours after treatment, and then from 24 to 36 hours after treatment. Cows had a similar pattern of decline in P4 concentration in the group treated through the IVG or i.m. route, and at 32 hours after

treatment both groups had mean P4 concentration below 1 ng/mL. Although this experiment also lacked a negative control group, results supported the hypothesis that luteal regression could be induced through IVG administration of PGF2α analogues. It is important to note that cows were at an unknown stage of the estrous cycle, and no individual P4 profiles were described, therefore it is possible that some cows had an immature CL at the time of treatment and did not undergo complete luteal regression. Another caveat of this experiment was that P4 concentration was only measured for up to 36 hours after treatment precluding drawing conclusions about complete luteal regression and circulating P4 levels at 56 to 72 hours after treatment which are approximate time points at which GnRH treatment and TAI take place in most synchronization of ovulation protocols.

Although available research regarding IVG administration of PGF2α in cattle is scarce and experiments present important limitations, available data supports the hypothesis that it is feasible to induce luteolysis through IVG delivery of PGF2α. Further research is required to describe in detail P4 dynamics after IVG administration of PGF2α in lactating dairy cows and to determine the efficacy of PGF2α to induce luteolysis when administered intravaginally.

5. Intravaginal administration of Gonadotropin releasing hormone

Gonadotropin releasing hormone is one of the main regulators of the reproductive physiology of cattle controlling the production and release of luteinizing hormone (LH) and primarily the synthesis of follicle stimulating hormone (FSH) (Millar, 2005). Therefore, as described in Chapter I of Section I, the availability of GnRH for its use in cattle provides veterinarians, producers, and researchers with an invaluable tool for controlling reproductive function in cattle.

Gonadotropin releasing hormone is a decapeptide molecule [pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH2; (Schally et al., 1971)] produced and stored in neurons from the medio-basal hypothalamus, and released by axon terminals in the median eminence of the brain (Sealfon et al., 1997). The most important structural features of the GnRH molecule are: (1) a bend in the Gly in position six, (2) presence of a NH_2 terminus (pGlu-His-Trp-Ser), and (3) the COOH terminus (Pro-Gly·NH2). These features are critical for receptor binding and response, thus amino acids substitutions in these portions will affect the potency of analogues and antagonists (Millar et al., 2004).

Substitutions of L-isomers with D-isomers in synthetic GnRH analogues provides them with either greater or lower affinity and activation capacity for the receptor as well as extended or reduced half-life (Padula, 2005). Several of these analogues and their potency were tested for their use in cattle to control reproductive cycles. The most widely used analogue is Gonadorelin (hereafter referred as GnRH) which is homologous to the natural hormone. Another commonly used analogue for reproductive management of cattle (not approved in the U.S.) is Buserelin. The latter is a nonapeptide lacking Gly in position 10 of the natural GnRH molecule and with a D-Ser substitution in position 6. These two modifications confer the molecule greater affinity for the GnRH receptor and an extended half-life (Karten and Rivier, 1986). In a randomized study, Chenault et al. (1990) compared the potency of GnRH and Buserelin. Heifers were treated with 10 or 20 µg of Buserelin or 150, 250, or 500 µg of GnRH between Days 8 and 16 of the estrous cycle. Both doses of Buserelin elicited an LH surge of greater magnitude (measured as area under the curve and maximum LH concentration) than the three different doses of GnRH, indicating a potency of approximately 50 times greater for Buserelin than Gonadorelin.

Although there are no reports in the literature regarding the use of the IVG route for the administration of GnRH or its analogues in cattle, there are several reports for other species such as rabbit (Viudes-De-Castro et al., 2007), swine (Stewart et al., 2010), and rats (Okada et al., 1982; Okada et al., 1983).

Viudes-De-Castro et al. (2007) performed two experiments to test the feasibility of inducing ovulation by including Buserelin or Triptorelin in the semen extender for intravaginal insemination in rabbit does. In the first experiment, they compared two different doses of Buserelin (2 and 5 µg) or Triptorelin (1 and 5 µg) to a negative control (no GnRH) and a positive control (1 µg Buserelin i.m.) using weaned does (n = 255). Animals were slaughtered three days after treatment and ovulatory response was measured by counting ovulatory stigmas. The positive control group had greater ovulatory response (97.8%) than the rest of the treatments, except than the group which received 2 µg of Buserelin through the IVG route (88.9%). Further, all groups receiving IVG Buserelin or Triptorelin had greater ovulatory response (70.0 to 88.9%) than the negative control group (32.5%). There was no difference in ovulatory response among IVG treatments. In a second experiment, does (n = 702) were inseminated and treated with 1 µg i.m. Buserelin or including 5 µg of Buserelin or 5 µg of Triptorelin in the semen diluent. There was no difference in pregnancy rate or kindling rate among treatments. Based on these results it was concluded that the inclusion of GnRH analogues in the semen diluent for IVG insemination can effectively induce ovulation in rabbits.

Stewart et al. (2010) carried out an experiment to evaluate the feasibility of inducing an LH surge and ovulation after IVG administration of Triptorelin with different concentrations of a methylcellulose gel in sows (n = 48). After weaning, sows were treated with 2 mL of 1.2% methylcellulose gel (negative control), or 100 µg of Triptorelin in 0.9, 1.2, or 1.5%

methylcellulose gel. The rationale for using different methylcellulose concentrations was that transmembrane transport efficiency increases when the vehicle and membrane viscosity are similar (Edwards et al., 1996). Blood samples were collected every 2 hours for 30 hours after treatment to measure LH concentration. Transabdominal ultrasonography was performed every 12 hours from 36 hours pretreatment until treatment, and every 4 hours thereafter until ovulation was confirmed. All sows receiving Triptorelin were inseminated 8 and 32 hours after treatment. Negative control and other 48 untreated sows were treated following conventional management practices consisting of insemination once a day for each day of estrus. Groups receiving Triptorelin in 1.2 and 1.5% methylcellulose gel had a significant increase in LH concentration 8 to 12 hours after treatment. Further, although there was no treatment effect on ovulatory rate, ovulations in sows receiving 1.2 and 1.5% methylcellulose plus Triptorelin occurred in a narrower period of time. No effect of treatment was observed on farrowing rate or number of piglets born alive per litter. These results showed that vaginal administration of GnRH analogues can induce an LH surge and ovulation in sows, and that viscosity of the vehicle has a direct impact on treatment efficacy.

Using rats in diestrus stage, Okada et al. (1982) compared the efficacy of intravenous, subcutaneous, nasal, oral, and intravaginal administration of Leuprolide (a GnRH analogue). For intravaginal administration both an aqueous and an oleaginous base including different types of organic acids were used. Leakage of Evan's blue into the vaginal mucosa after intravenous administration was used as a measure of membrane permeability. When using ovulatory response after treatment as the outcome of interest, the IVG route of delivery showed the greatest potency among all parenteral routes of administration and no difference was observed between the aqueous and the oleaginous vehicle. Moreover, addition of organic acids to the IVG

preparation increased the ovulatory response. Relative potency was maximum when adding 10% citric acid (relative potency = 4.9), 10% succinic acid (relative potency = 5.4), and glycholic acid (relative potency = 5.6). These acids also increased staining of the vaginal mucosa with Evan's blue, suggesting increased permeability of the vaginal mucosa. In a second experiment, Okada et al. (1983) evaluated the effect of adding different concentrations of citric acid and different pH on permeability of the vaginal mucosa and ovulatory response to IVG administration of Leuprolide to rats in diestrus. Decreasing pH from 6.7 to 2.02 resulted in a fourfold increase in ovulatory response. Ovulatory response increased with concentration of citric acid in the vehicle, reaching maximum response at 7 to 10%. Further, addition of 5% citric acid at pH 3.5 and 1.8 resulted in a threefold increase of potency. These two experiments together provided evidence that the addition of citric acid as an absorption enhancer and acidification of the vehicle increased permeability of the vaginal mucosa to Leuprolide resulting in a greater ovulatory response in rats.

Although these experiments were not performed in cattle, they provide strong evidence that the vagina is a feasible route for GnRH administration. Data also suggest that due to the low lipophilicity of GnRH and its analogues, the use of vehicles that facilitate transmembrane transport should be used for IVG administration of GnRH. Further research in cattle is required to determine the feasibility of inducing an LH surge and ovulation through IVG administration of GnRH.

6. Summary

The objective of this literature review was to summarize important aspects regarding the potential use of the vagina as a hormone delivery route for synchronization of ovulation programs and current knowledge regarding the use of PGF2α and GnRH through the IVG route.

Due to its anatomy and vascularization, the vagina is a practical and effective route for hormone delivery in several species. Its anatomy allows holding hormone delivery devices for prolonged periods of time and it is currently used for P4 administration in cattle. Although research regarding IVG administration of PGF2α in cattle is limited and published experiments have experimental design limitations, most data support the idea that it is feasible to induce luteolysis through IVG administration of PGF2α analogues.

Additionally, there is a substantial amount of published research from non-cattle species supporting the feasibility of using the IVG route for delivery of GnRH analogues. Conversely, to my knowledge no data from experiments with cattle have been reported.

Thus, the objective of the experiments presented in Chapters II and III of Section II was to test the feasibility of using the IVG route for delivery of PGF2α and GnRH in dairy cattle. In the experiments presented in Chapter II, we tested the feasibility of inducing luteal regression after IVG administration of PGF2α in lactating dairy cows. The objective of the experiments presented in Chapter III was to test the feasibility of inducing an LH surge of similar magnitude of that induced after i.m. administration of GnRH using the IVG route.

7. References

Blazquez, N., E. Batten, S. Long, G. Perry, and O. Whelehan. 1989. A quantitative morphological study of the bovine vaginal epithelium during the oestrous cycle. Journal of comparative pathology 100:187-193.

Borchardt, S., P. Haimerl, A. Pohl, and W. Heuwieser. 2017. Evaluation of prostaglandin F 2α versus prostaglandin F 2α plus gonadotropin-releasing hormone as Presynch methods preceding an Ovsynch in lactating dairy cows: A meta-analysis. Journal of Dairy Science 100:4065-4077.

Bourne, G., S. Moss, P. Phillips, and B. Shuker. 1980. The metabolic fate of the synthetic prostaglandin cloprostenol ('Estrumate') in the cow: Use of ion cluster techniques to facilitate metabolite identification. Biological Mass Spectrometry 7:226-230.

Chenault, J., D. Kratzer, R. Rzepkowski, and M. Goodwin. 1990. LH and FSH response of Holstein heifers to fertirelin acetate, gonadorelin and buserelin. Theriogenology 34:81-98.

Cole, H. 1930. A study of the mucosa of the genital tract of the cow, with special reference to the cyclic changes. American Journal of Anatomy 46:261-301.

Cross, P. S., R. Künnemeyer, C. R. Bunt, D. A. Carnegie, and M. J. Rathbone. 2004. Control, communication and monitoring of intravaginal drug delivery in dairy cows. International journal of pharmaceutics 282:35-44.

Dukes, M., W. Russell, and A. Walpole. 1974. Potent luteolytic agents related to prostaglandin F2α.

Dyce, K. M., W. O. Sack, and C. J. G. Wensing. 2009. Textbook of veterinary anatomy. Elsevier Health Sciences.

Edwards, D. A., K. J. Gooch, I. Zhang, G. H. McKinley, and R. Langer. 1996. The nucleation of receptor-mediated endocytosis. Proceedings of the National Academy of Sciences of the United States of America 93:1786-1791.

Flynn, G., N. Ho, S. Hwang, E. Owada, A. Molokhia, C. Behl, W. Higuchi, T. Yotsuyanagi, Y. Shah, and J. Park. 1976. Interfacing Matrix Release and Membrane Absorption-Analysis of Steroid Absorption from a Vaginal Device in the Rabbit Doe. ACS Publications.

Fricke, P., D. Caraviello, K. Weigel, and M. Welle. 2003. Fertility of dairy cows after resynchronization of ovulation at three intervals following first timed insemination. Journal of dairy science 86:3941-3950.

Funk, C. D. 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294:1871-1875.

Galvão, K. and J. Santos. 2010. Factors affecting synchronization and conception rate after the Ovsynch protocol in lactating Holstein cows. Reproduction in domestic animals 45:439-446.

Giordano, J., M. Wiltbank, J. Guenther, R. Pawlisch, S. Bas, A. Cunha, and P. Fricke. 2012. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. Journal of dairy science 95:639-653.

Heinonen, K., T. Shiferaw, and M. Heinonen. 1996. Oestrous synchronisation in Ethiopian highland zebu cattle by means of intravaginal cloprostenole administration. Tropical animal health and production 28:121-125.

Hussain, A. and F. Ahsan. 2005. The vagina as a route for systemic drug delivery. Journal of controlled release 103:301-313.

Hwang, S., E. Owada, T. Yotsuyanagi, L. Suhardja, N. Ho, G. Flynn, and W. Higuchi. 1976. Systems approach to vaginal delivery of drugs II: In situ vaginal absorption of unbranched aliphatic alcohols. Journal of pharmaceutical sciences 65:1574-1578.

Karsch, F. J., J. M. Bowen, A. Caraty, N. P. Evans, and S. M. Moenter. 1997. Gonadotropin-releasing hormone requirements for ovulation. Biology of reproduction 56:303-309.

Karten, M. J. and J. E. RIVIER. 1986. Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: rationale and perspective. Endocrine Reviews 7:44-66.

Kimball, F., J. Lauderdale, N. Nelson, and R. Jackson. 1976. Comparison of luteolytic effectiveness of several prostaglandin analogs in heifers and relative binding affinity for bovine luteal prostaglandin binding sites. Prostaglandins 12:985-995.

Künnemeyer, R., P. Cross, C. Bunt, D. Carnegie, and M. Rathbone. 2004. Electronically controlled, intravaginal drug delivery. Proceedings of the Institution of Mechanical Engineers, Part B: Journal of Engineering Manufacture 218:1409-1415.

Lamb, G., C. Dahlen, J. Larson, G. Marquezini, and J. Stevenson. 2010. Control of the estrous cycle to improve fertility for fixed-time artificial insemination in beef cattle: a review. Journal of animal science 88:E181-E192.

Macmillan, K. and A. Peterson. 1993. A new intravaginal progesterone releasing device for cattle (CIDR-B) for oestrous synchronisation, increasing pregnancy rates and the treatment of post-partum anoestrus. Animal Reproduction Science 33:1-25.

Macmillan, K., V. Taufa, D. Barnes, and A. Day. 1991. Plasma progesterone concentrations in heifers and cows treated with a new intravaginal device. Animal Reproduction Science 26:25-40.

Macmillan, K. L. 2010. Recent advances in the synchronization of estrus and ovulation in dairy cows. Journal of reproduction and development 56:S42-S47.

Manns, J. and H. Hafs. 1976. Controlled breeding in cattle: A review. Canadian Journal of Animal Science 56:121-130.

Millar, R. P. 2005. GnRHs and GnRH receptors. Animal reproduction science 88:5-28.

Millar, R. P., Z.-L. Lu, A. J. Pawson, C. A. Flanagan, K. Morgan, and S. R. Maudsley. 2004. Gonadotropin-releasing hormone receptors. Endocrine reviews 25:235-275.

Moreira, F., C. Orlandi, C. Risco, R. Mattos, F. Lopes, and W. Thatcher. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. Journal of Dairy Science 84:1646-1659.

Niswender, G. D., J. L. Juengel, P. J. Silva, M. K. Rollyson, and E. W. McIntush. 2000. Mechanisms controlling the function and life span of the corpus luteum. Physiological reviews 80:1-29.

Okada, H., I. Yamazaki, Y. Ogawa, S. Hirai, T. Yashiki, and H. Mima. 1982. Vaginal absorption of a potent luteinizing hormone-releasing hormone analog (leuprolide) in rats I: Absorption by various routes and absorption enhancement. Journal of pharmaceutical sciences 71:1367-1371.

Okada, H., I. Yamazaki, T. Yashiki, and H. Mima. 1983. Vaginal absorption of a potent luteinizing hormone-releasing hormone analogue (leuprolide) in rats II: Mechanism of absorption enhancement with organic acids. Journal of Pharmaceutical Sciences 72:75-78.

Owada, E., C. Behl, S. Hwang, L. Suhardja, G. Flynn, N. Ho, and W. Higuchi. 1977. Vaginal drug absorption in rhesus monkeys I: Development of methodology. Journal of pharmaceutical sciences 66:216-219.

Padula, A. 2005. GnRH analogues—agonists and antagonists. Animal reproduction science 88:115-126.

Pursley, J., M. Mee, and M. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF 2α and GnRH. Theriogenology 44:915-923.

Pursley, J., M. Wiltbank, J. Stevenson, J. Ottobre, H. Garverick, and L. Anderson. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus. Journal of Dairy Science 80:295-300.

Rathbone, M. J. and C. R. Burke. 2013. Controlled Release Intravaginal Veterinary Drug Delivery. Pages 247-270 in Long Acting Animal Health Drug Products. Springer.

Reeves, P. R. 1978. Distribution, elimination, and residue studies in the cow with the synthetic prostaglandin estrumate. Journal of agricultural and food chemistry 26:152-155.

Richardson, J. L. and L. Illum. 1992. (D) Routes of delivery: Case studies:(8) The vaginal route of peptide and protein drug delivery. Advanced drug delivery reviews 8:341-366.

Schally, A., A. Arimura, Y. Baba, R. Nair, H. Matsuo, T. Redding, L. Debeljuk, and W. White. 1971. Isolation and properties of the FSH and LH-releasing hormone. Biochemical and biophysical research communications 43:393-399.

Schilling, E. and J. Zust. 1968. Diagnosis of oestrus and ovulation in cows by pH-measurements intra vaginam and by apparent viscosity of vaginal mucus. Journal of reproduction and fertility 15:307-311.

Sealfon, S. C., H. Weinstein, and R. P. Millar. 1997. Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. Endocrine reviews 18:180-205.

Shrestha, H. K., M. A. Beg, R. R. Burnette, and O. Ginther. 2012. Plasma clearance and half-life of prostaglandin F2alpha: a comparison between mares and heifers. Biology of reproduction 87:18, 11-16.

Sørensen, L. P., M. Bjerring, and P. Løvendahl. 2016. Monitoring individual cow udder health in automated milking systems using online somatic cell counts. Journal of dairy science 99:608-620.

Stangaferro, M., R. Wijma, L. Caixeta, M. Al-Abri, and J. Giordano. 2016. Use of rumination and activity monitoring for the identification of dairy cows with health disorders: Part I. Metabolic and digestive disorders. Journal of Dairy Science 99:7395-7410.

Stevenson, J. and A. Phatak. 2005. Inseminations at estrus induced by presynchronization before application of synchronized estrus and ovulation. Journal of dairy science 88:399-405.

Stewart, K., W. Flowers, G. Rampacek, D. Greger, M. Swanson, and H. Hafs. 2010. Endocrine, ovulatory and reproductive characteristics of sows treated with an intravaginal GnRH agonist. Animal reproduction science 120:112-119.

Viudes-de-Castro, M., R. Lavara, F. Marco-Jiménez, C. Cortell, and J. Vicente. 2007. Ovulation induced by mucosa vaginal absorption of buserelin and triptorelin in rabbit. Theriogenology 68:1031-1036.

Webel, S. 1976. Control of the estrous cycle in cattle with a progesterone releasing intravaginal device. Pages 521-523 in Proc. Proceedings of the 8th International Congress on Animal Reproduction and Artificial Insemination.

Wiltbank, M. C. and J. R. Pursley. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. Theriogenology 81:170-185.

Zdunczyk, S., P. Andresen, T. Janowski, and A. Chmielewski. 1994. [The effectiveness of intravaginal administration of estrumate for cows in silent heat in relation to the progesterone concentration in the blood]. DTW. Deutsche tierarztliche Wochenschrift 101:440-441.

CHAPTER II

CIRCULATING PROGESTERONE DYNAMICS AFTER INTRAVAGINAL INSTILLATION OF PROSTAGLANDIN-F2α TO LACTATING DAIRY COWS

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ABSTRACT

Our objectives were to evaluate circulating progesterone (P4) concentration dynamics and test the feasibility of inducing luteal regression after intravaginal (IVG) instillation of the PGF2α analogue Dinoprost (PGF) in lactating dairy cows. In two experiments, cows were synchronized using the Ovsynch protocol to induce the formation of a corpus luteum (CL). Cows with at least one functional (P4 ≥1 ng/mL) CL ≥15 mm 7.5 d after Ovsynch remained in the studies. In Exp 1, cows (n = 31) were stratified by parity group and received: 5 mL of saline IVG (SAL-IVG, n = 6), 25 mg of PGF2α i.m. (PGF25-IM, n = 7), 25 mg of PGF2α IVG (PGF25-IVG, n = 6), 50 mg of PGF2α IVG (PGF50-IVG, n = 6), and 125 mg of PGF2α IVG (PGF125-IVG, n = 6). Experiment 2 was conducted to test the hypothesis that IVG instillation of two 25 mg doses of PGF2α 12 h apart would be more effective than a 25 or 50 mg dose in a single application. Cows (n = 32) were stratified by parity and received: SAL-IVG (n = 7), PGF25-IM (n = 7), PGF25-IVG (n = 6), and PGF50-IVG (n = 6) as in Exp 1, whereas another group received two IVG instillations of 25 mg of PGF2α 12 h apart (PGF25-2X-IVG, n = 6). Blood was collected at -1 h, every 6 h from 0 h to 24 h, and every 12 h up to 96 h after treatment (trt). In Exp 1, there was an effect of trt (P <0.01), time (P <0.001), and an interaction between trt and time on P4 concentrations (P <0.001). All PGF-treated groups had lower (P <0.05)

concentrations of P4 than cows in the SAL-IVG group from 12 to 96 h after trt. Although an initial decline in P4 concentrations was induced in all PGF-treated cows, some cows in the IVG-treated groups presented a rebound in plasma P4, indicating CL recovery. More cows in the PGF25-IVG and PGF125-IVG groups than in the PGF50-IVG and PGF25-IM groups presented CL recovery, suggesting that greater doses of PGF2α may not necessarily improve CL regression. In Exp. 2, there was an effect of trt ($P < 0.001$), time ($P < 0.001$), and an interaction between trt and time on P4 concentrations ($P < 0.001$). All PGF-treated groups had lower ($P < 0.05$) P4 than the SAL-IVG group from 12 to 96 h after trt. Cows in the PGF25-2X-IVG group had a P4 profile that was similar to that of cows in the PGF25-IM group and the lowest P4 concentrations after treatment among the IVG-treated groups, and all cows presented complete CL regression (defined as P4 <0.4 ng/mL). We conclude that CL regression can be induced through IVG instillation of PGF2α in lactating dairy cows and that instillation of two IVG doses of 25 mg of PGF2α 12 h apart was the most effective strategy.

INTRODUCTION

Reproductive efficiency in cattle is critical to farm success, notably affecting farm profitability (De Vries, 2006; Giordano et al., 2011; Rodgers et al., 2012). In this regard, synchronization of ovulation protocols for timed AI (TAI) are essential to reproductive management in modern cattle operations, assuring timely insemination and greatly improving fertility (Wiltbank and Pursley, 2014). Also, synchronization protocols can serve as a therapeutic approach for treating specific physiological limitations, or can be adapted to suit the physiological status of individual cows (Stevenson et al., 2006; Bisinotto et al., 2015; Giordano et al., 2015). The need to provide multiple hormone injections is a major barrier to adoption of

TAI, with some protocols requiring up to six injections over a period of 35 days. Many dairy and beef farms in the U.S. and across the world are unable to take advantage of TAI because they lack the necessary facilities, tools, qualified labor, or the frequency of access to cows that is necessary for properly implementing a protocol. For current users, issues regarding labor efficiency and animal welfare are also becoming more important, as protocols continue to evolve to include more hormone injections at inconvenient frequencies.

Thus, developing devices that allow cattle operations to fully automate hormone administration for synchronization of ovulation could be a solution to these major barriers to adoption of TAI programs. Automating hormone administration could also create opportunities for developing novel protocols and optimizing current ones to maximize fertility by closely mimicking or enhancing physiological processes. A potential alternative to automated hormone administration for synchronization of estrus and ovulation is intravaginal (IVG) administration of hormones through electronically controlled devices (Rathbone et al., 1998; Cross et al., 2004; Künnemeyer et al., 2004). Nevertheless, feasibility data to support IVG administration of some of the most widely used reproductive hormones (PGF2α and GnRH) for synchronization of estrus and ovulation in cattle is lacking.

The vagina presents favorable attributes as a route of hormone administration; it is easily accessible, the mucosa is densely vascularized, and it is highly permeable to molecules with low molecular weight (Okada et al., 1982; Hussain and Ahsan, 2005). In cattle, the use of the IVG route for administration of reproductive hormones has been limited to sustained progesterone (P4) release over prolonged periods of time (≥ 7 d) (Macmillan et al., 1991; Macmillan and Peterson, 1993; Chebel et al., 2006). Conversely, little is known about hormones used to elicit an immediate response through im injection such as PGF2α and GnRH. For example, the efficacy

of PGF2α in inducing luteolysis after IVG instillation has been studied very rarely. To the best of our knowledge, only two studies have tested the efficacy of IVG treatment with PGF2α to induce luteolysis in cattle (Zdunczyk et al., 1994; Heinonen et al., 1996). These studies provided some evidence to support the feasibility of using IVG PGF2α to induce luteal regression but they were inconclusive because only estrus expression in small groups of cows (Heinonen et al., 1996) or estrus expression and concentrations of P4 36 h after treatment were evaluated (Zdunczyk et al., 1994). Thus, our objective is to characterize and optimize the response of cattle to IVG treatment with PGF2α to facilitate the future development of automated IVG hormone delivery devices for synchronization of ovulation in cattle.

We conducted two experiments to study P4 concentration dynamics after IVG instillation of PGF2α 7.5 d following induction of ovulation in lactating dairy cows. In Experiment 1, our objectives were to compare P4 concentration dynamics after instillation of varying doses of PGF. Our specific hypothesis was that IVG instillation of PGF2α would induce luteal regression and the changes in circulating P4 would be similar to those observed after an i.m. injection of PGF. In addition, we hypothesized that the response to IVG instillation of PGF2α would be dose-dependent so that greater doses would be more effective than lower doses at reducing circulating concentrations of P4. Based on the results of Experiment 1, we conducted Experiment 2 to test the hypothesis that instillation of two IVG doses of 25 mg of PGF2α 12 h apart would be more effective than instillation of a single IVG dose of 25 or 50 mg of PGF, and would be similar to an i.m. injection of 25 mg of PGF2α for reducing circulating P4 concentrations. Thus, the objectives of Experiment 2 were to evaluate P4 concentration dynamics after instillation of two IVG doses of 25 mg PGF2α 12 h apart.

MATERIALS AND METHODS

Animals

Lactating Holstein cows from the dairy unit of the Cornell University Ruminant Center (Harford, NY) were used for this experiment conducted from November 2013 to October 2014. Cows were housed in free-stall barns with cooling fans placed above the feeding lane and free stalls with sprinklers above the feed bunk. All cows were fed a TMR diet once daily and had ad libitum access to feed and water. The diet was formulated to meet or exceed nutritional requirements for lactating Holstein cows producing 45 kg of milk based on the Cornell Net Carbohydrate and Protein System version 6.5 (CNCPS). Cows were milked thrice daily at ~8 h intervals and received bovine somatotropin (Posilac, 500 mg; Elanco Animal Health, Indianapolis, IN) at 14 d intervals beginning ~60 DIM until dried off. All procedures were approved by the Animal Care and Use Committee of Cornell University.

Treatments

In Experiments 1 (Exp 1) and 2 (Exp 2), lactating non-pregnant cows at various DIM received the Ovsynch-56 protocol (Brusveen et al., 2009) with two PGF2α injections (GnRH-7 d-PGF-1 d-PGF-32 h-GnRH) to synchronize ovulation. At the time of the second GnRH injection of Ovsynch and 48 h later, transrectal ovarian ultrasonography (TUS) was performed using a portable ultrasound machine with a 7.5 MHz linear array transducer (IbexPro, E.I Medical Imaging, Colorado, USA) to confirm ovulation after GnRH. Ovulation was defined as the disappearance of at least one follicle ≥10 mm and the presence of a putative CL on the same ovary. Seven and a half days after the second GnRH injection of Ovsynch, cows that ovulated were re-examined by TUS and only those with a CL ≥ 15 mm remained in the study (Figure 2.1). Plasma concentrations of P4 were used to confirm that the CL was functional (P4 >1 ng/mL) in

all cows that received the treatments. For synchronization of ovulation with the Ovsynch protocol and the respective PGF2α treatments, Dinoprost tromethamine (Lutalyse, Zoetis Animal Health, New York, NY) was used, whereas the GnRH product was Gonadorelin diacetate tetrahydrate (Cystorelin, Merial LLC, Duluth, GA).

Experiment 1*:* Cows (15 primiparous and 24 multiparous) with average DIM of 420 (range 124 to 772) and average BCS of 3.9 (range 3.5 to 4.5) were used for this experiment. After confirmation of the presence of a CL ≥ 15 mm 7.5 d after induction of ovulation, cows (15 primiparous and 16 multiparous) were stratified by parity group (primiparous vs. multiparous) and randomly assigned to one of five treatments: 1) 5 mL of saline solution IVG (SAL-IVG, n = 6), 2) an i.m. injection of 25 mg of PGF2α in the semimembranosus or semitendinosus muscle (PGF25-IM, n = 7), 3) 25 mg of PGF2α IVG (PGF25-IVG, n = 6), 4) 50 mg of PGF2α IVG (PGF50-IVG, n = 6), and 5) 125 mg of PGF2α IVG (PGF125-IVG, n = 6). Seven cows had two corpora lutea present and were randomly assigned to the PGF2α treatment groups only (two cows in groups PGF25-IM, PGF25-IVG, and PGF50-IVG and one cow in the PGF125-IVG group).

Experiment 2*:* Cows (16 primiparous and 23 multiparous), with average DIM of 124 (range 51 to 740) and average BCS of 3.1 (range 2.5 to 4.75) were used for this experiment. After confirmation of the presence of a CL ≥ 15 mm 7.5 d after induction of ovulation, cows (15 primiparous and 17 multiparous) were stratified by parity group and randomly assigned to one of five treatments: 1) 5 mL of saline solution IVG (SAL-IVG, n = 7), 2) an i.m. injection of 25 mg of PGF2α in the semimembranosus or semitendinosus muscle (PGF25-IM, n = 7), 3) 25 mg of

PGF2α IVG (PGF25-IVG, n = 6), 4) 50 mg of PGF2α IVG (PGF50-IVG, n = 6), and 6) two doses of 25 mg of PGF2α IVG administered 12 h apart (PGF25-2X-IVG, n = 6). Two cows had two corpora lutea present and were randomly assigned to the PGF2α treatment groups only (one cow each in groups PGF25-IM and PGF50-IVG).

In both experiments cows received the IVG instillations of PGF2α or saline following the same procedures while cows were restrained in self-locking headgates after their morning milking. The vulva was manually cleaned and disinfected using chlorhexidine solution and dried off using paper towels. After cleaning the vulva, the vulvar labia were manually opened by one technician while another technician inserted a uterine infusion catheter (44.5 cm long by 0.5 cm of external diameter) attached to a plastic syringe. The catheter was carefully inserted into the vagina until the cervix or vaginal fornix was reached. Once in the cranial portion of the vagina, the catheter was pulled backwards 1 to 2 cm and the volume of saline solution or PGF2α for the specific treatment was released. After treatment, cows remained standing in self-locking headgates for a period of 45 to 60 minutes to record urination activity, backflow of fluid from the vagina, and signs of distress. Fresh TMR was offered to cows during the observation period.

Blood sampling and determination of progesterone concentrations

Blood samples were obtained from the coccygeal vein or artery using evacuated tubes containing sodium heparin (Vacutainer, BD, Franklin Lakes, NJ). Blood samples were collected at -1 h, 0 h, every 6 h for the first 24 h, and then every 12 h up to 96 h after treatment (Figure 2.1). Samples were immediately placed into crushed ice and centrifuged within 2 h at 2000 x g for 20 minutes at 4°C. Plasma was harvested and stored at -20°C until assayed for P4. Plasma concentrations of P4 were determined in duplicate using a commercial solid-phase, no-extraction

radioimmunoassay (Coat-a-count; Diagnostic Products Corp., Los Angeles, CA). In the nine assays performed two quality control samples with concentrations of P4 representative of the diestrus (high concentration = 7.4 ng/mL) and proestrus (low concentration = 0.5 ng/mL) phases of the estrous cycle were included twice (at the beginning and end) across each assay to determine the variation within and across assays. Average sensitivity for the nine P4 assays was 0.03 ng/mL. The average intra-assay CV for the high-concentration sample was 5.7% whereas the interassay CV was 9.3%. For the low-concentration sample the intra-assay CV was 5.7% whereas the inter-assay CV was 14.9%.

For these experiments, the presence of an active or functional CL was defined as circulating P4 concentrations ≥1 ng/mL (Ginther et al., 2010), whereas complete luteal regression was defined as circulating P4 concentrations <0.4 ng/mL (Carvalho et al., 2015) for at least one sample collected after treatment.

Statistical Analysis

Data for Exp 1 and Exp 2 were analyzed following the same methodology. Before statistical analysis, normality of data for concentrations of P4 was assessed using the Shapiro-Wilk statistic and graphical methods (histogram and Q-Q plot) with PROC UNIVARIATE of SAS (version 9.4, SAS Institute Inc., Cary, NC). In addition, during statistical analysis normality of residuals was assessed using graphical methods generated with the residual option of PROC MIXED of SAS. Because all methodologies indicated that values for concentrations of P4 were not normally distributed, square root transformation was used to correct for non-normality. After transformation, the square root of the concentration of P4 was analyzed by ANOVA with repeated measures using PROC MIXED of SAS. A spatial power covariance structure was used

to adjust for the varying time intervals at which blood was collected. Treatment, time, and treatment-by-time interaction were included as fixed effects, whereas cows within the treatment group were used as a random effect in the models. Parity group (primiparous vs. multiparous) was also offered to each model as a categorical fixed effect. The Least Significant Difference (LSD) post-hoc mean separation test was used to determine differences between Least Square Means (LSM).

An analysis similar to that conducted for Exp 1 and Exp 2 was performed by combining data from both experiments. Four of the treatments (SAL-IVG, PGF25-IM, PGF25-IVG, and PGF50-IVG) were identical in both experiments, whereas the PGF125-IVG and PGF125-2X-IVG treatments were included in Exp 1 and Exp 2, respectively, but not in both. Both experiments were conducted in the same facilities, by the same technicians, using cows from the same herd, and were conducted seven months apart.

In addition, the combined data set was used to evaluate concentrations of P4 at 48, 60, and 72 h after treatment. Differences in concentrations of P4 at 48, 60, and 72 h were determined by ANOVA with PROC MIXED of SAS with a model that contained treatment as the sole explanatory variable. The LSD post-hoc mean separation test was used to determine differences between LSMs.

For clarity of presentation all values are reported as arithmetical means and SE generated with PROC MEANS of SAS, whereas proportions were generated using PROC FREQ of SAS. All explanatory variables and their interactions were considered significant if $P \leq 0.05$, whereas $0.05 < P \leq 0.10$ were considered a tendency.

RESULTS

During the observation period following application of the experimental treatments, none of the cows in the treatment groups urinated or presented backflow of fluid from the vagina. Also, no signs of distress or abnormalities in behavior were observed.

Experiment 1: Progesterone concentration dynamics

From -1 to 96 h after treatment, there was an effect of treatment ($P < 0.01$), time ($P < 0.001$), and an interaction between treatment and time ($P < 0.001$) on circulating concentrations of P4 (Figure 2.2A). At 6 h after treatment cows in the PGF50-IVG group presented lower ($P < 0.05$) concentrations of P4 than cows in the SAL-IVG group (negative control), whereas cows in the PGF25-IM, PGF25-IVG, and PGF125-IVG groups tended ($P < 0.10$) to have lower concentrations of P4 than cows in the SAL-IVG group. From 12 to 96 h after treatment, cows in all PGF-treated groups had lower ($P < 0.05$) concentrations of P4 than cows in the SAL-IVG group. Concentrations of P4 were similar ($P > 0.05$) for all PGF-treated groups during the entire sampling period, except that cows in the PGF25-IM group had lower P4 concentrations than cows in the PGF25-IVG group at 96 h. Parity did not affect ($P = 0.46$) circulating concentrations of P4.

Experiment 2: Progesterone concentration dynamics

From -1 to 96 h after treatment, there was an effect of treatment ($P < 0.001$), time ($P < 0.001$), and an interaction between treatment and time ($P < 0.001$) on circulating concentrations of P4 (Figure 2.2B). At 6 h after treatment, cows in the PGF25-2X-IVG group had lower ($P < 0.05$) whereas cows in the PGF25-IM group tended ($P < 0.10$) to have lower concentrations of P4 than cows in the SAL-IVG group. From 12 to 96 h after treatment, cows in all PGF-treated

groups had lower (P <0.05) concentrations of P4 than cows in the SAL-IVG group. Parity tended to affect (P = 0.07) circulating concentrations of P4 (overall mean = 1.4 ± 0.1 vs. 1.3 ± 0.1 ng/mL for primiparous and multiparous, respectively).

Also, concentrations of P4 differed across the PGF-treated groups (Figure 2.2B). At 48 h cows in the PGF25-2X-IVG and PGF25-im groups tended (P <0.10) to have lower concentrations of P4 and from 60 to 96 h after treatment had lower (P <0.05) concentrations of P4 than cows in the PGF25-IVG group. At 84 and 96 h after treatment, cows in the PGF25-2X-IVG group tended (P <0.10) to have lower concentrations of P4 than cows in the PGF50-IVG group, whereas cows in the PGF25-IM group tended (P <0.10) to have lower concentrations of P4 than cows in the PGF50-IVG group only at 96 h after treatment.

Experiment 1 and Experiment 2 combined: Progesterone dynamics

From -1 to 96 h after treatment, there was an effect of treatment (P <0.001), time (P <0.001), and an interaction between treatment and time (P <0.001) on circulating concentrations of P4 (Figure 2.3). From 6 to 96 h after treatment, concentrations of P4 were lower (P < 0.05) for cows in all the PGF-treated groups than for cows in the SAL-IVG group. At 60 h after treatment, cows in the PGF25-2X-IVG group had lower (P< 0.05) concentrations of P4 than cows in the PGF25-IVG, PGF50-IVG, and PGF125-IVG groups, a difference that persisted up to 96 h with the PGF25-IVG group only (Figure 2.3). A similar pattern was observed for cows in the PGF25-IM and PGF25-IVG groups, except that at 60 h only a tendency was detected (Figure 2.3). Parity did not affect (P = 0.67) circulating concentrations of P4.

Progesterone concentration patterns and luteal regression for cows in Experiment 1 and Experiment 2 combined

Among cows treated with PGF, three distinct subgroups were identified based on their P4 concentration patterns. A subgroup of cows presented the expected pattern of P4 decline after treatment [79% (11/14), 42% (5/12), 50% (6/12), 67% (4/6), and 100% (6/6) of cows in the PGF25-IM, PGF25-IVG, PGF50-IVG, PGF125-IVG and PGF25-2X-IVG groups, respectively]. These cows had an 81% to 99% reduction in concentrations of P4 by 36 h after the PGF2α treatment. Their plasma concentrations of P4 were below the cutoff value that indicates the presence of an active CL (1 ng/mL) by 36 h after treatment and all but one cow (P4 = 0.43 ng/mL) had P4 concentrations indicating complete luteal regression (<0.4 ng/mL) by 72 h after treatment (Figure 2.4A). Thereafter, concentrations of P4 continued to decline or remained steady but did not increase above 1 ng/mL (indicating the presence of active CL) up to the end of the sampling period (0.04 to 0.61 ng/mL). Another subgroup of cows from the PGF25-IVG [17% (2/12)], PGF50-IVG [50% (6/12)], and PGF25-IM [7% (1/14)] groups had an initial 56% to 91% reduction in concentrations of P4 from -1 to 36 h after treatment, reaching nadir concentrations of P4 of 0.30 to 0.94 ng/mL (Figure 2.4B). However, by 96 h after treatment, concentrations of P4 were above 1 ng/mL (1.75 to 4.55 ng/mL), indicating the presence of an active CL and lack of complete luteal regression (<0.4 ng/mL). Finally, a third subgroup of cows [14% (2/14), 41% (5/12), and 33% (2/6), of the cows in the PGF25-IM, PGF25-IVG, and PGF125-IVG treatment groups, respectively] presented an initial 26% to 74% decline in concentrations of P4, reaching a nadir during the first 36 h after treatment. In these cows, concentrations of P4 were never below the cutoff that indicates the presence of an active CL (1 ng/mL) or complete luteal regression (0.4 ng/mL). The nadir was followed by a substantial increase in concentrations of P4, reaching

up to >100% of their original concentrations of P4 by 96 h after treatment (2.45 to 9.98 ng/mL; Figure 2.4C).

Concentrations of progesterone at 48, 60, and 72 h after treatment

Concentrations of P4 at 48, 60, and 72 h after treatment are presented in Table 2.1. At the three time points evaluated, concentrations of P4 were greater ($P < 0.05$) for cows in the SAL-IVG group than for cows in all the PGF-treated groups. Concentrations of P4 for cows in the PGF25-2X-IVG group were lower ($P < 0.05$) than for cows in the PGF25-IVG group at the three time points of interest. For cows in the PGF25-IM group, concentrations of P4 were lower ($P < 0.05$) than for cows in the PGF25-IVG group at 48 and 72 h after treatment. Concentrations of P4 for cows in the PGF50-IVG and PGF125-IVG groups were similar to those of cows in the PGF25-IVG, PGF25-2X-IVG, and PGF25-IM groups at the three time points evaluated.

Table 2.1. Concentrations of progesterone at 48, 60, and 72 h after treatment for cows in Experiments 1 and 2 combined.

	Time after treatment (h)		
Treatment[1]	48	60	72
	P4[2] (ng/mL)	P4 (ng/mL)	P4 (ng/mL)
SAL-IVG	5.96 ± 0.53[a]	6.26 ± 0.54[a]	5.99 ± 0.47[a]
PGF25-IM	0.63 ± 0.27[c]	0.67 ± 0.23[bc]	0.57 ± 0.24[c]
PGF25-IVG	1.55 ± 0.42[b]	1.74 ± 0.50[b]	2.24 ± 0.64[b]
PGF50-IVG	0.83 ± 0.25[bc]	0.79 ± 0.24[bc]	1.13 ± 0.40[bc]
PGF125-IVG	1.81 ± 1.01[bc]	2.15 ± 1.35[bc]	1.65 ± 0.98[bc]
PGF25-2X-IVG	0.27 ± 0.07[c]	0.19 ± 0.04[c]	0.21 ± 0.07[c]

[1]Cows received the following treatments 7.5 d after the second GnRH injection of the Ovsynch protocol: SAL-IVG = 5 mL of saline IVG (n = 13), PGF25-IM = i.m. injection of 25 mg of PGF2α (n = 14), PGF25-IVG = 25 mg of PGF2α IVG (n = 12), PGF50-IVG = 50 mg of PGF2α IVG (n = 12), PGF125-IVG = 125 mg of PGF2α IVG (n = 6), and PGF25-2X-IVG = two IVG doses of 25 mg of PGF,12 h apart (n = 6).abcDifferent superscripts within a colum differ statisctically (P < 0.05). 2P4 = progesterone.

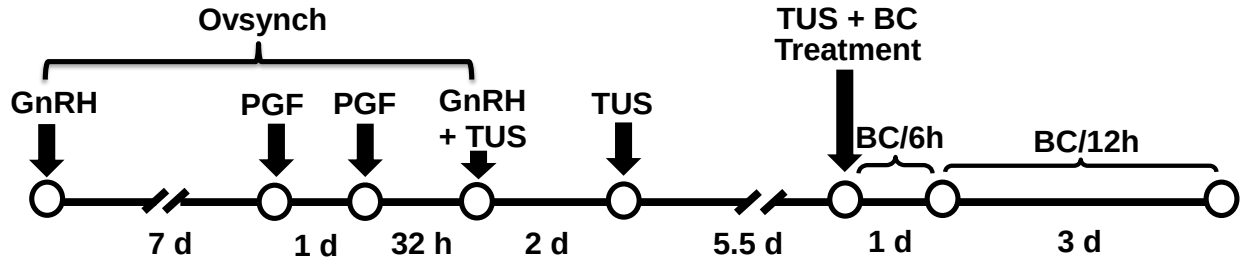
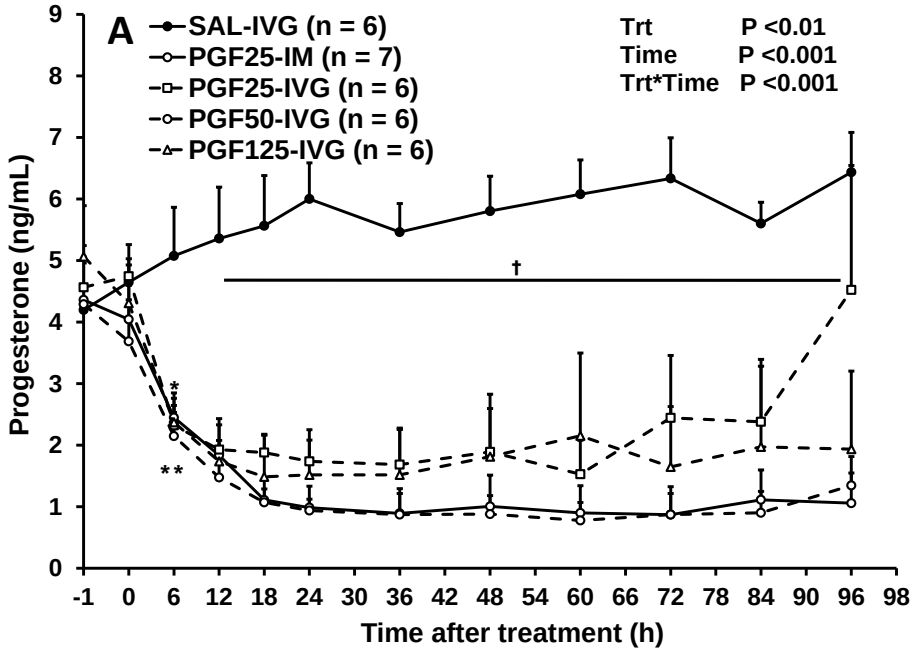
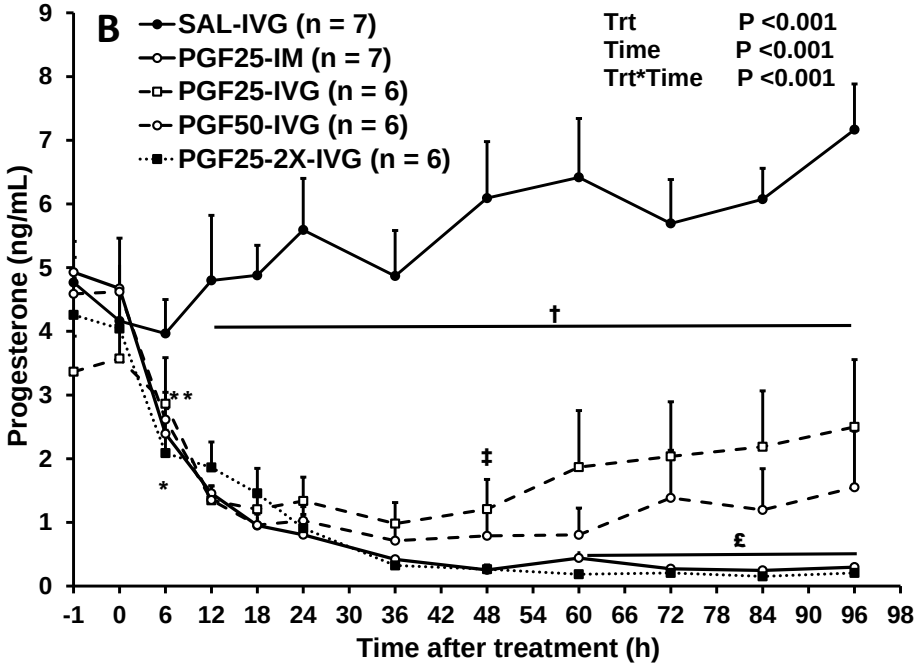


Figure 2.1 Cows were synchronized using the Ovsynch protocol with two PGF2α injections (GnRH-7 d-PGF-1 d-PGF-32 h-GnRH). Cows with at least one functional (P4 ≥1 ng/mL) CL ≥15 mm 7.5 d after the second GnRH injection of Ovsynch remained in the study to receive the treatments. In Exp 1, cows (n = 31) stratified by parity received: 5 mL of saline solution IVG (SAL-IVG, n = 6), an i.m. injection of 25 mg of PGF2α (PGF25-IM, n = 7), 25 mg of PGF2α IVG (PGF25-IVG, n = 6), 50 mg of PGF2α IVG (PGF50-IVG, n = 6), and 125 mg of PGF2α IVG (PGF125-IVG, n = 6). In Exp 2, cows (n = 32) stratified by parity received: SAL-IVG (n = 7), PGF25-IM (n = 7), PGF25-IVG (n = 6), PGF50-IVG (n = 6) as in Experiment 1, and another group of two IVG doses of 25 mg of PGF2α 12 h apart (PGF25-2X-IVG, n = 6). Blood was collected at -1 h, 0 h, every 6 h up to 24 h, and then every 12 h up to 96 h after treatment. TUS = transrectal ultrasonography, BC = blood collection.

Figure 2.2. (A) Circulating concentrations of progesterone (P4) from -1 to 96 h after treatment for Experiment 1. Seven and a half days after the second GnRH injection of the Ovsynch protocol, cows with at least one functional (P4 ≥1 ng/mL) CL ≥15 mm received the following treatments: SAL-IVG = 5 mL of saline IVG (n = 6), PGF25-IM = an i.m. injection of 25 mg of PGF2α (n = 7), PGF25-IVG = 25 mg of PGF2α IVG (n = 6), PGF50-IVG = 50 mg of PGF2α IVG (n = 6), and PGF125-IVG = 125 mg of PGF2α IVG (n = 6). There was an effect of treatment ($P < 0.01$), time ($P < 0.001$), and an interaction between treatment and time ($P < 0.001$) on circulating concentrations of P4. * = concentrations of P4 for cows in the PGF25-IM, PGF25-IVG, and PGF125-IVG groups tended ($P < 0.10$) to be lower than for cows in the SAL-IVG group. ** = concentrations of P4 for cows in the PGF50-IVG group were lower ($P < 0.05$) than for cows in the SAL-IVG group. † = concentrations of P4 were lower ($P < 0.05$) for cows in all PGF2α treatment groups than for cows in the SAL-IVG group. Concentrations of P4 were similar ($P > 0.05$) for cows in all PGF-treated groups during the entire sampling period, except that cows in the PGF25-IM group differed from cows in the PGF25-IVG group at 96 h.
(B) Circulating concentrations of P4 from -1 to 96 h after treatment for Experiment 2. Cows were set up as described for Experiment 1 and received the following treatments: SAL-IVG = 5 mL of saline solution IVG (n = 7), PGF25-IM = an i.m. injection of 25 mg of PGF2α (n = 7), PGF25-IVG = 25 mg of PGF2α IVG (n = 6), PGF50-IVG = 50 mg of PGF2α IVG (n = 6), and PGF25-2X-IVG = two IVG doses of 25 mg of PGF2α administered 12 h apart (n = 6). There was an effect of treatment ($P < 0.001$), time ($P < 0.001$), and an interaction between treatment and time ($P < 0.001$) on circulating concentrations of P4. * = concentrations of P4 for cows in the PGF25-IM group tended ($P < 0.10$) to be lower than for cows in the SAL-IVG group. ** = concentrations of P4 for cows in the PGF25-2X-IVG group were lower ($P < 0.05$) than for cows in the SAL-IVG group. † = concentrations of P4 for cows in all PGF2α treated groups were lower ($P < 0.05$) than for cows in the SAL-IVG group. ‡ = concentrations of P4 at 48 h tended ($P < 0.10$) to be lower for cows in groups PGF25-2X-IVG and PGF25-IM than for cows in the PGF25-IVG group. £ = concentrations of P4 from 60 to 96 h were lower ($P < 0.05$) for cows in groups PGF25-2X-IVG and PGF25-IM than for cows in the PGF25-IVG group. Also, at 84 and 96 h, concentrations of P4 for cows in the PGF25-2X-IVG group tended ($P < 0.10$) to be lower than for cows in the PGF50-IVG group. At 96 h concentrations of P4 for cows in the PGF25-IM group tended ($P < 0.10$) to be lower than for cows in the PGF50-IVG group.


A
SAL-IVG (n = 6)
PGF25-IM (n = 7)
PGF25-IVG (n = 6)
PGF50-IVG (n = 6)
PGF125-IVG (n = 6)
Trt P <0.01
Time P <0.001
Trt*Time P <0.001
Progesterone (ng/mL)
Time after treatment (h)
†
*
**


B
SAL-IVG (n = 7)
PGF25-IM (n = 7)
PGF25-IVG (n = 6)
PGF50-IVG (n = 6)
PGF25-2X-IVG (n = 6)
Trt P <0.001
Time P <0.001
Trt*Time P <0.001
Progesterone (ng/mL)
Time after treatment (h)
†
‡
£
*
**

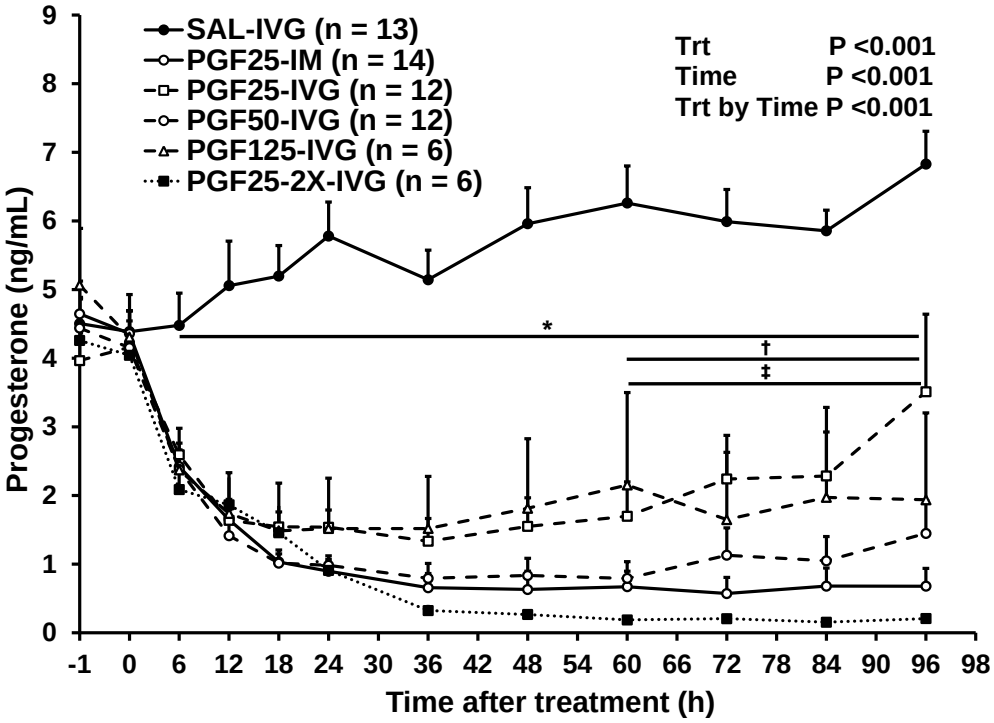
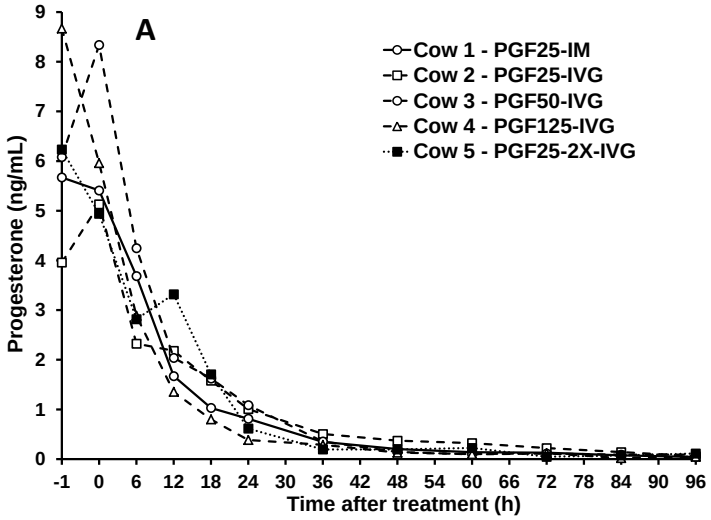
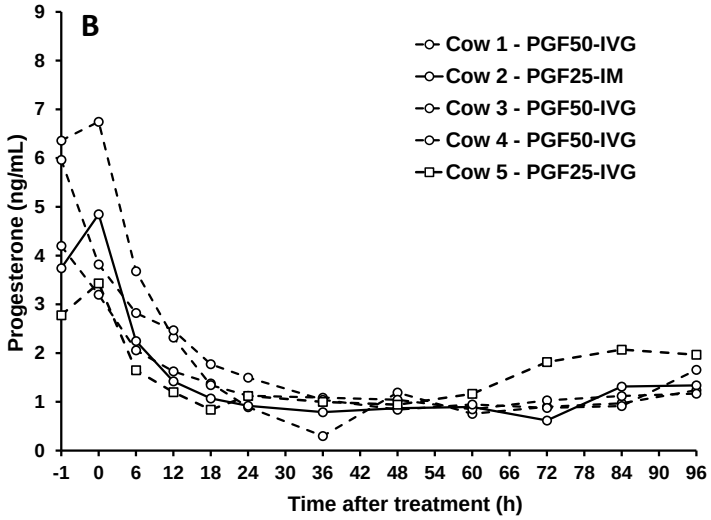
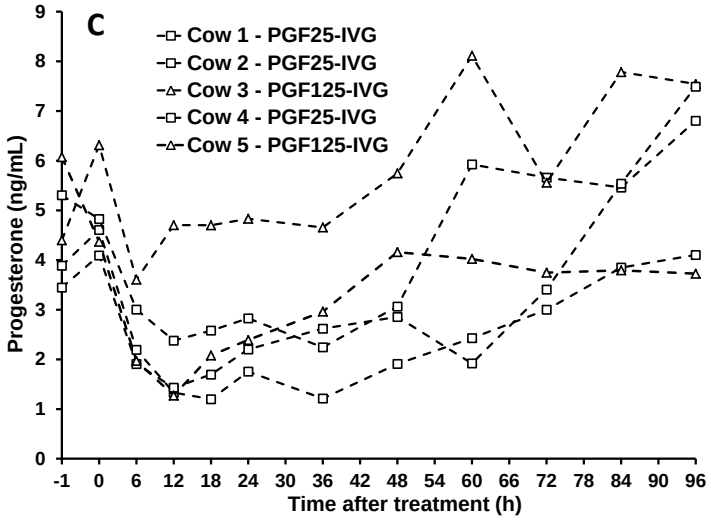


Figure 2.3. Circulating concentrations of Progesterone (P4) from 1 h before to 96 h after treatment for Experiments 1 and 2 combined. Cows with at least one functional (P4 ≥1 ng/mL) CL ≥15 mm received the following treatments 7.5 d after the second GnRH injection of the Ovsynch protocol: SAL-IVG = 5 mL of saline IVG (n = 13), PGF25-IM = an i.m. injection of 25 mg of PGF2α (PGF; n = 14), PGF25-IVG = 25 mg of PGF2α IVG (n = 12), PGF50-IVG = 50 mg of PGF2α IVG (n = 12), PGF125-IVG = 125 mg of PGF2α IVG (n = 6), and PGF25-2X-IVG = two IVG doses of 25 mg of PGF2α administered 12 h apart (n = 6). There was an effect of treatment (P <0.01), time (P <0.001), and an interaction between treatment and time (P <0.001) on circulating concentrations of P4. * = cows in the SAL-IVG groups were different (P <0.05) from cows in all other treatments. † = at 60 h, cows in group PGF25-2X-IVG had lower (P <0.05) concentrations of P4 than cows in groups PGF25-IVG, PGF50-IVG, and PGF125-IVG, a difference that persisted up to 96 h for cows in the PGF25-2X-IVG and PGF25-IVG groups only. ‡ = a tendency (P <0.10) at 60 h and then lower (P <0.05) concentrations of P4 up to 96 h was observed for cows in group PGF25-IM than for cows in the PGF25-IVG, PGF50-IVG, and PGF125-IVG groups.

Figure 2.4. Individual plasma progesterone (P4) profiles of representative cows within subgroups, based on concentration of P4 patterns after receiving experimental treatments. (A) Cows with the expected pattern of P4 decline after the PGF treatment. These cows presented an 81% to 99% reduction in concentration of P4 during the first 36 h after treatment and reached the cutoff value for complete luteal regression (P4 <0.4 ng/mL) by 72 h in all but one cow, which reached a nadir of 0.43 ng/mL of P4. (B) Cows with an initial 56% to 91% reduction in concentrations of P4 from -1 to 36 h after treatment that reached nadir concentrations of P4 of 0.30 to 0.94 ng/mL. However, by 96 h after treatment, concentrations of P4 were above 1 ng/mL (1.75 to 4.55 ng/mL), indicating the presence of an active CL and lack of complete luteal regression (<0.4 ng/mL). (C) Cows with an initial decline in P4 concentrations of 26% to 74% followed by a substantial increase in P4 concentrations up to >100% of their original concentrations by 96 h after treatment (2.45 to 9.98 ng/mL). Concentrations of P4 were never below the cutoff indicating the presence of an active CL (1 ng/mL) or complete luteal regression (0.4 ng/mL).


A
Cow 1 - PGF25-IM
Cow 2 - PGF25-IVG
Cow 3 - PGF50-IVG
Cow 4 - PGF125-IVG
Cow 5 - PGF25-2X-IVG
Progesterone (ng/mL)
Time after treatment (h)


B
Cow 1 - PGF50-IVG
Cow 2 - PGF25-IM
Cow 3 - PGF50-IVG
Cow 4 - PGF50-IVG
Cow 5 - PGF25-IVG
Progesterone (ng/mL)
Time after treatment (h)


C
Cow 1 - PGF25-IVG
Cow 2 - PGF25-IVG
Cow 3 - PGF125-IVG
Cow 4 - PGF25-IVG
Cow 5 - PGF125-IVG
Progesterone (ng/mL)
Time after treatment (h)

DISCUSSION

The results of our experiments support the initial hypothesis that IVG administration of PGF can successfully induce luteal regression in cattle. An initial decline in concentrations of P4 indicating induction of luteolysis was evident for cows in all PGF-treated groups during the first 12 to 24 h after treatment in both experiments. Nevertheless, complete CL regression was not observed for all cows in all treatments. In all PGF-treated groups there were cows that presented complete luteal regression (P4 <0.4 ng/mL), but some cows (from all groups except those in the IVG25-2X-IVG group) presented a substantial initial decline in plasma P4 followed by a major increase in P4 production. These findings suggest that luteolysis was initiated but the CL was able to recover its functionality. This phenomenon was more prevalent for cows in the PGF25-IVG and PGF125-IVG groups. Although failure to induce complete luteal regression is commonly observed in ~10% to 28% of lactating dairy cows after an i.m. injection of the labeled dose of PGF in the U.S. (Brusveen et al., 2009; Ribeiro et al., 2012; Giordano et al., 2013), based on the observed pattern of P4 production after treatment and the high percentage of cows (33% to 50%) failing to complete luteolysis after the single IVG instillation of 25 to 125 mg of PGF, it appears that this treatment strategy may not be as effective as a single i.m. injection of 25 mg of PGF.

It is uncertain at the moment which factors are responsible for the incomplete luteal regression and rebound in P4 production by the CL. A better response to the 50 than the 25 mg dose of PGF would suggest that absorption of insufficient amounts of PGF from the vagina may have been a leading factor in the failed response. Nevertheless, the poor overall response to the 125 mg dose of PGF and the fact that at least some cows from the two higher-dose treatments failed to complete luteal regression does not support that notion. Indeed, our hypothesis that higher doses of PGF would be more effective than lower doses was not fully supported. While

we observed that the 50 mg dose of PGF was more effective than a 25 mg dose, the 125 mg dose of PGF did not further reduce concentrations of P4. Future research should include larger numbers of cows to confirm that higher doses of PGF are less effective in inducing complete CL regression than lower doses. Moreover, estimations of IVG PGF absorption through determination of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM; metabolite of $PGF_{2\alpha}$) in circulation could help determine whether absorption of PGF limits the response to the higher-dose treatments.

Our results from Exp 2 strongly support our hypothesis that two IVG instillations of 25 mg of PGF 12 h apart should further reduce concentrations of P4 when compared to single IVG treatments. As opposed to the treatment groups that received similar or higher doses of PGF through IVG instillation, the two instillations 12 h apart resulted in mean concentrations of P4 <0.4 ng/mL from 48 to 96 h after treatment and complete CL regression in 100% of the cows. Unlike cows in the other groups that received IVG PGF, none of the cows in the PGF25-2X-PGF group presented a rebound in P4 production of sufficient magnitude to indicate full CL recovery (P4 $\geq$ 1 ng/mL). Some of the cows (3/6) presented a rebound in P4 concentrations (0.5 to 2.5 ng/mL range) within the first 24 h after treatment, but the second PGF treatment blunted the rebound and these cows successfully completed luteolysis (data not shown). In spite of the reduced scale of our studies for determining the efficacy of IVG instillation of PGF for complete luteal regression, our current results are encouraging. Additional research with sufficient numbers of cows to validly test the hypothesis that two IVG treatments of PGF is as effective as a single i.m. injection of PGF at the dose used in synchronization of estrus and ovulation protocols is warranted.

It is certainly possible that the rebound in circulating concentrations of P4 observed in some cows may have resulted from premature ovulation before the end of the sampling period. We cannot rule out this possibility because we did not monitor estrous behavior or ovulation; however, we speculate that it is rather unlikely because all cows had a functional CL at the time of treatment and in most of these cows P4 never declined below 1 ng/mL.

Even though very limited data are available about P4 concentration dynamics and luteal regression after IVG instillation of PGF in cattle, our current results agree with those of a previous study that documented a decline in concentrations of P4 after IVG instillation of a 500 µg dose of the PGF analogue Cloprostenol in lactating dairy cows with functional CLs (Zdunczyk et al., 1994). Unlike our study, Zdunczyk et al. (1994) reported complete luteal regression (defined as P4 <1 ng/mL) in all treated cows (12/12) by 32 h after treatment. A caveat of their study was that cows were monitored for only 36 h after treatment, precluding the detection of rebounds in concentrations of P4 similar to those observed in our study and others in which cows failed to complete luteal regression (Ginther et al., 2009; Nascimento et al., 2014). Differences in cows (breed, physiological status), experimental conditions, or the PGF analogue used may also be plausible explanations for the differences between the two studies' findings. Interestingly, Zdunczyk et al. (1994) also reported that 66.6% of cows (8/12) that received PGF through IVG instillation presented estrous behavior within 10 d after treatment, compared with 70% of cows (7/10) receiving the same dose through i.m. injection. Despite the limited number of cows, this study provided partial evidence that cows may undergo complete luteal regression and express estrous behavior after IVG instillation of PGF. A second set of studies conducted with non-lactating Zebu cattle of unknown cyclicity status documented estrous behavior after

IVG instillation of a 175 µg dose of Cloprostenol; however, concentrations of P4 were not monitored (Heinonen et al., 1996).

In one experiment without a control group, 61.5% (24/39) of cows that received PGF through IVG instillation were detected as being in estrus within three days of treatment and 66.7% (16/24) of the inseminated cows became pregnant. In a second study in which cows received an i.m. injection or IVG instillation of Cloprostenol, the estrus response determined by visual observation or rectal palpation was 62.5% (25/40) and 60.6% (20/33) for the IVG and i.m. groups, respectively. Interestingly, these data may support the notion that IVG instillation of Cloprostenol is an effective strategy for inducing luteal regression because the estrus response fell within the expected range for cattle treated with PGF (Walker et al., 1996) but involved a 65% lower dose than the luteolytic dose used in synchronization of estrus and ovulation protocols (500 µg). Whether Cloprostenol can be used at very low doses or is more effective than Dinoprost for causing luteal regression after IVG instillation is unknown and warrants further investigation.

Collectively, these previous studies and our current research, which has documented in detail the dynamics of concentration of P4 after instillation of PGF at varying doses and frequencies, provide evidence that the IVG instillation of PGF may be effective in inducing luteal regression in cattle. Furthermore, the improved response observed after IVG instillation of two 25 mg doses of PGF 12 h apart suggests that such a treatment strategy may be the most suitable alternative for testing the efficacy of IVG instillation of PGF for synchronizing estrus and ovulation protocols in large field studies.

Because 48 and 60 h after PGF treatment are the approximate time intervals at which cows enrolled in Ovsynch-type protocols would receive the last GnRH injection of the protocol

and 72 h is the approximate time of insemination, we evaluated concentrations of P4 at these time points. This is relevant because low circulating concentrations of P4 at the time of a GnRH injection are critical for inducing an effective LH surge and subsequent ovulation in dairy cattle (Walker et al., 1996; Ginther et al., 2009). As expected, cows in the SAL-IVG group (negative control) had greater concentrations of P4 than cows in all the PGF-treated groups at the time points of interest because the CL remained functional (P4 ≥ 1 ng/mL) in all cows. Conversely, either partial (presenting an initial P4 decline but where P4 is never <0.4 ng/mL) or complete (with P4 <0.4 ng/mL) luteal regression was induced in all cows in the PGF-treated groups. Also, the fact that concentrations of P4 for cows in the PGF25-2X-IVG group were similar to what we observed for cows in the PGF25-IM group and lower than for cows in the PGF25-IVG group at all time points evaluated reinforces the notion that the PGF25-2X-IVG treatment may be as effective as the i.m. treatment and more effective than the single IVG treatment with 25 mg of Dinoprost. Remarkably, concentrations of P4 for cows in the PGF25-2X-IVG group were below 1.0 ng/mL by 36 h after treatment and continued to decline to levels (<0.4 ng/mL) known to be optimal for maximal fertility in lactating dairy cows receiving TAI (Carvalho et al., 2015). The lack of difference between the PGF25-IM and PGF25-2X-IVG groups and the PGF50-IVG and PGF125-IVG groups is likely the result of a Type II error because of the limited number of cows per group and the substantial variation in P4 concentrations due to incomplete luteal regression in some cows.

CONCLUSION

Our studies confirm the feasibility of inducing luteal regression through IVG instillation of PGF in lactating dairy cattle. Our research indicates that two instillations of 25 mg of PGF 12

h apart may be an effective strategy for inducing complete luteal regression. Unlike the other groups receiving PGF through IVG instillation, there was no CL recovery in any of the cows receiving the two instillations after induction of luteolysis. Further research with a greater number of cows is needed to determine the best IVG instillation regimens for causing complete luteal regression in synchronization of ovulation protocols in cattle.

Despite the feasibility of inducing luteal regression through IVG instillation of PGF that we demonstrated in these experiments, our research should not be used as evidence to support or recommend the use of IVG instillation of PGF in commercial cattle operations because it constitutes off-label use of PGF products.

REFERENCES

Bisinotto, R., L. Castro, M. Pansani, C. Narciso, N. Martinez, L. Sinedino, T. Pinto, N. Van de Burgwal, H. Bosman, and R. Surjus. 2015. Progesterone supplementation to lactating dairy cows without a corpus luteum at initiation of the Ovsynch protocol. Journal of dairy science 98:2515-2528.

Brusveen, D., A. Souza, and M. Wiltbank. 2009. Effects of additional prostaglandin F 2α and estradiol-17β during Ovsynch in lactating dairy cows. Journal of dairy science 92:1412-1422.

Carvalho, P., A. Souza, M. Wiltbank, and P. Fricke. 2015. Progesterone concentration at each treatment during an Ovsynch protocol affects fertility to timed AI in lactating Holstein cows. Journal of dairy science 98:92.

Chebel, R., J. Santos, R. Cerri, H. Rutigliano, and R. Bruno. 2006. Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. Journal of dairy science 89:4205-4219.

Cross, P. S., R. Künnemeyer, C. R. Bunt, D. A. Carnegie, and M. J. Rathbone. 2004. Control, communication and monitoring of intravaginal drug delivery in dairy cows. International journal of pharmaceutics 282:35-44.

De Vries, A. 2006. Economic value of pregnancy in dairy cattle. Journal of dairy science 89:3876-3885.

Ginther, O., R. Araujo, M. Palhao, B. Rodrigues, and M. Beg. 2009. Necessity of Sequential Pulses of Prostaglandin F2alpha for Complete Physiologic Luteolysis in Cattle 1. Biology of reproduction 80:641-648.

Ginther, O., H. Shrestha, M. Fuenzalida, A. Shahiduzzaman, M. Hannan, and M. Beg. 2010. Intrapulse temporality between pulses of a metabolite of prostaglandin F 2α and circulating concentrations of progesterone before, during, and after spontaneous luteolysis in heifers. Theriogenology 74:1179-1186.

Giordano, J., P. Fricke, M. Wiltbank, and V. Cabrera. 2011. An economic decision-making support system for selection of reproductive management programs on dairy farms. Journal of dairy science 94:6216-6232.

Giordano, J., M. Stangaferro, R. Wijma, W. Chandler, and R. Watters. 2015. Reproductive performance of dairy cows managed with a program aimed at increasing insemination of cows in

estrus based on increased physical activity and fertility of timed artificial inseminations. Journal of dairy science 98:2488-2501.

Giordano, J. O., M. C. Wiltbank, P. M. Fricke, S. Bas, R. Pawlisch, J. N. Guenther, and A. B. Nascimento. 2013. Effect of increasing GnRH and PGF 2α dose during Double-Ovsynch on ovulatory response, luteal regression, and fertility of lactating dairy cows. Theriogenology 80:773-783.

Heinonen, K., T. Shiferaw, and M. Heinonen. 1996. Oestrous synchronisation in Ethiopian highland zebu cattle by means of intravaginal Cloprostenole administration. Tropical animal health and production 28:121-125.

Hussain, A. and F. Ahsan. 2005. The vagina as a route for systemic drug delivery. Journal of controlled release 103:301-313.

Künnemeyer, R., P. Cross, C. Bunt, D. Carnegie, and M. Rathbone. 2004. Electronically controlled, intravaginal drug delivery. Proceedings of the Institution of Mechanical Engineers, Part B: Journal of Engineering Manufacture 218:1409-1415.

Macmillan, K. and A. Peterson. 1993. A new intravaginal progesterone releasing device for cattle (CIDR-B) for oestrous synchronisation, increasing pregnancy rates and the treatment of post-partum anoestrus. Animal Reproduction Science 33:1-25.

Macmillan, K., V. Taufa, D. Barnes, and A. Day. 1991. Plasma progesterone concentrations in heifers and cows treated with a new intravaginal device. Animal Reproduction Science 26:25-40.

Nascimento, A. B., A. H. Souza, A. Keskin, R. Sartori, and M. C. Wiltbank. 2014. Lack of complete regression of the Day 5 corpus luteum after one or two doses of PGF 2α in nonlactating Holstein cows. Theriogenology 81:389-395.

Okada, H., I. Yamazaki, Y. Ogawa, S. Hirai, T. Yashiki, and H. Mima. 1982. Vaginal absorption of a potent luteinizing hormone-releasing hormone analog (leuprolide) in rats I: Absorption by various routes and absorption enhancement. Journal of pharmaceutical sciences 71:1367-1371.

Rathbone, M. J., K. L. Macmillan, K. Inskeep, S. Burggraaf, and C. R. Bunt. 1998. Fertility regulation in cattle. Journal of controlled release 54:117-148.

Ribeiro, E., R. Bisinotto, M. Favoreto, L. Martins, R. Cerri, F. Silvestre, L. Greco, W. Thatcher, and J. Santos. 2012. Fertility in dairy cows following presynchronization and administering

twice the luteolytic dose of prostaglandin F 2α as one or two injections in the 5-day timed artificial insemination protocol. Theriogenology 78:273-284.

Rodgers, J., S. Bird, J. Larson, N. DiLorenzo, C. Dahlen, A. DiCostanzo, and G. Lamb. 2012. An economic evaluation of estrous synchronization and timed artificial insemination in suckled beef cows. Journal of animal science 90:4055-4062.

Stevenson, J., J. Pursley, H. Garverick, P. Fricke, D. Kesler, J. Ottobre, and M. Wiltbank. 2006. Treatment of cycling and noncycling lactating dairy cows with progesterone during Ovsynch. Journal of dairy science 89:2567-2578.

Walker, W., R. Nebel, and M. McGilliard. 1996. Time of ovulation relative to mounting activity in dairy cattle. Journal of dairy science 79:1555-1561.

Wiltbank, M. C. and J. R. Pursley. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. Theriogenology 81:170-185.

Zdunczyk, S., P. Andresen, T. Janowski, and A. Chmielewski. 1994. Studies on intravaginal applications of Estrumate (R) in silent heat cows with emphasis of progesterone concentration in blood. Deutsche tierarztliche wochenschrift 101:440-441.

CHAPTER III

INTRAVAGINAL INSTILLATION OF GNRH ANALOGUES WITH AN ABSORPTION ENHANCER INDUCED A SURGE OF LH IN LACTATING DAIRY COWS

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ABSTRACT

Our objectives were to evaluate circulating LH concentrations after intravaginal (IVG) instillation of GnRH analogues in lactating dairy cows. In two experiments, lactating Holstein cows (Experiment 1: n = 32; Experiment 2: n = 47) received the experimental treatments 48 h after the first of two PGF2α treatments given 12 h apart and 7 d after a modified Ovsynch protocol (GnRH-7 d-PGF2α-24 h-PGF2α-56 h-GnRH). In Experiment 1, cows were stratified by parity and randomly allocated to receive the following treatments: 2 mL of saline IVG (SAL, n = 6), 100 µg of Gonadorelin (Gon) i.m. (G100-IM, n = 5), and 100 (G100, n = 7), 500 (G500, n = 8), or 1,000 µg of Gon IVG (G1000, n = 7). In Experiment 2, treatments were: 2 mL of saline IVG (SAL, n = 8), 100 µg of Gon i.m. (G100-IM, n = 8), 1,000 µg of Gon IVG (G1000, n = 7), 1,000 µg of Gon plus 10% citric acid (CA) IVG (G1000CA, n = 8), 80 µg of Buserelin IVG (B80, n = 8), and 80 µg of Buserelin plus 10% CA IVG (B80CA, n = 8). In both experiments, blood was collected every 15 min from -15 min to 4 h, and every 30 min from 4 to 6 h after treatment. Data for area under the curve (AUC), mean LH concentrations, and time to maximum LH concentration were analyzed by ANOVA with (mean LH only) or without repeated measures using PROC MIXED of SAS. The proportion of cows with a surge of LH was evaluated with

Fisher's exact test using PROC FREQ of SAS. In both experiments LH concentrations were affected by treatment, time, and the treatment by time interaction. In Experiment 1, the AUC for LH and maximum LH concentration were greatest for the G100-IM treatment and were greater for the G1000 than for the SAL and G500 treatments. The proportion of cows with an observed surge of LH was 100% and 0% for cows that received Gon i.m. and IVG, respectively. In Experiment 2, the AUC and maximum LH concentrations were greater for the G100-IM, G1000CA, and B80CA treatments than for the other IVG treatments. The proportion of cows with a surge of LH differed by treatment (SAL = 0%, G100-IM = 100%, G1000 = 14%, G1000CA = 88%, B80 = 13% and B80CA = 100%). For the treatments with a surge of LH, time to maximum concentration of LH was the shortest for the G100-IM treatment, intermediate for the G1000CA treatment, and the longest for cows in the B80CA treatment. In conclusion, Gonadorelin (up to 1,000 µg) absorption through intact vaginal epithelium after a single IVG instillation was insufficient to elicit a surge of LH of normal magnitude. Conversely, IVG instillation of 1,000 µg of Gonadorelin and 80 µg of Buserelin with the addition of citric acid as absorption enhancer resulted in a surge of LH of similar characteristics than that induced after i.m. injection of 100 µg of Gonadorelin.

Keywords: intravaginal, GnRH analogue, citric acid, LH surge

INTRODUCTION

Timed artificial insemination (TAI) is one of the most widely used biotechnologies in cattle operations around the world (Lamb et al., 2010; Wiltbank and Pursley, 2014). In dairy farms, systematic implementation of synchronization of ovulation protocols ensures timely

insemination (Pursley et al., 1997; Fricke et al., 2003) and improves fertility outcomes (Moreira et al., 2001; Souza et al., 2008; Giordano et al., 2012b). Synchronization of ovulation protocols are continuously evolving to optimize follicle development, luteal regression, timing of ovulation, and the endocrine environment before and after TAI. As a result, groups of cows must receive hormonal treatments on multiple days of the week and different times of the day which may reduce protocol compliance and success in farms without appropriate facilities, frequent access to animals, and availability of qualified labor. Frequent cow manipulation also disrupts time budgets and normal behavior. Thus, fully automated hormone delivery systems may be an alternative to individual injections for facilitating implementation and improving compliance with synchronization of ovulation protocols. Automating hormone delivery may also allow designing more effective treatments and/or treatments tailored to individual or subgroups of cows based on their physiological or health status.

Ideally, the body cavity to insert and hold an automated hormone delivery system should allow holding the device for prolonged periods of time and easy access to the device for removal after use. Because the vagina meets these criteria, fully automated electronically-controlled intravaginal (IVG) drug delivery devices have been described (Rathbone et al., 1998; Cross et al., 2004; Künnemeyer et al., 2004) and could be developed for use in cattle. Another important attribute of a body cavity to insert an automated hormone delivery device is to allow proper absorption of hormones to elicit the desired physiological response. Although sustained release of progesterone (P4) through non-automated delivery devices has been extensively studied and is currently used in synchronization of estrus and ovulation protocols (Macmillan et al., 1991; Macmillan and Peterson, 1993; Chebel et al., 2006), the feasibility of IVG administration of important reproductive hormones such as, GnRH and PGF2α, has been rarely studied and IVG

administration is not currently used in cattle. Beyond potential differences in molecular structure that may affect IVG absorption, a fundamental difference between IVG treatments with P4 and hormones like GnRH and PGF2α is that the former can effectively exert its biological function through sustained release because an acute effect is not necessary. Conversely, administration of exogenous GnRH is only effective to induce a surge of LH and PGF2α to trigger luteolysis through immediate absorption and an acute effect on their target tissues (i.e., pituitary gland for GnRH and corpus luteum for PGF2α).

The feasibility of inducing a surge of LH of similar magnitude after IVG than i.m. administration of GnRH or its analogues has been previously studied in other species such as the sow (Stewart et al., 2010), the rat (Okada et al., 1982; 1983), and the rabbit (Viudes-de-Castro et al., 2007) but not in cattle. Although GnRH absorption through epithelial walls can occur through transmembrane diffusion, vesicle receptor mediated transport, or para cellular diffusion (Richardson and Illum, 1992), vaginal absorption may be challenging. Transmembrane transport is probably limited due to the hydrophilic nature of GnRH and a receptor mediated transport mechanism in the vaginal epithelium seems unlikely. Further, para cellular transport may be limited by inter cellular apical junction complexes (Hussain and Ahsan, 2005) formed by tight junctions and adherens junctions (Ivanov et al., 2005).

To overcome challenges associated with absorption efficiency of molecules through intact tissues, absorption enhancers can be included to disrupt the integrity of intercellular junctions so that para cellular transport increases (Okada et al., 1982; 1983; Fatakdawala and Uhland, 2011). For example, using a rat model, Okada et al. (1982, 1983) observed an increase in absorption and vaginal permeability to Leuprolide (i.e., a GnRH analogue) when including citric acid in the vehicle. Carboxylic acids, such as citric acid, chelate calcium which has been

shown to loosen intercellular tight junctions, thus facilitating intercellular transport of molecules (Cho et al., 1989). In addition to absorption enhancers, a potential strategy to increase the efficacy of hormones administered through the IVG route may be the use of more potent hormone analogues. Although absorption of all analogues may be equally compromised, more potent analogues could be more effective because smaller amounts are needed to elicit a satisfactory physiological response. In the case of GnRH, multiple analogues of varying potency are available. For example, Buserelin has been shown to be up to 50 times more potent than Gonadorelin (Chenault et al., 1990; Picard-Hagen et al., 2015), thereby Buserelin may be an alternative to Gonadorelin for IVG administration.

We performed two experiments to evaluate the feasibility of inducing a surge of LH after IVG instillation of GnRH analogues in lactating dairy cows. We aimed to determine if it was possible to induce a surge of LH of similar magnitude, timing, and duration after IVG instillation of GnRH analogues than after i.m. injection of the labeled dose of Gonadorelin to induce ovulation in cattle (i.e., 100 µg). Specifically, the objective of Experiment 1 was to compare circulating LH concentrations after IVG instillation of different doses of the GnRH analogue Gonadorelin. We hypothesized that IVG instillation of Gonadorelin would induce a surge of LH similar to that observed after i.m. injection of 100 µg of Gonadorelin. Also, we hypothesized that instillation of greater doses of Gonadorelin would result in greater circulating LH concentrations. Based on the results of Experiment 1, we conducted a second experiment to evaluate LH concentrations after IVG instillation of Gonadorelin or Buserelin with or without the inclusion of citric acid as an absorption enhancer. We hypothesized that the inclusion of citric acid to the GnRH solution and the use of a GnRH analog of greater potency than Gonadorelin (i.e.,

Buserelin) would result in a surge of LH similar to that observed after i.m. injection of 100 µg of Gonadorelin.

MATERIALS AND METHODS

Animals

All procedures were approved by the Animal Care and Use Committee of Cornell University.

Lactating Holstein cows from the dairy unit of the Cornell University Ruminant Center (Harford, NY) were used for these experiments conducted from October 2014 to March 2016. Cows were housed in free stall barns up to the day before intensive blood sample collection when they were moved to a tie-stall barn. Free-stall barns were equipped with deep bedded sand stalls, cooling fans placed above the feeding lane and free-stalls, and sprinklers above the feed bunk. The tie-stall barn was equipped with deep bedded wood dust stalls, individual waterers, feed bins, and tunnel ventilation. All cows were fed a TMR diet once daily and had ad libitum access to feed and water. The diet was formulated to meet or exceed nutritional requirements for lactating Holstein cows producing 45 kg of milk based on the Cornell Net Carbohydrate and Protein System version 6.5. Cows were milked thrice daily at ~ 8 h intervals and received bovine somatotropin (Posilac, 500 mg; Elanco Animal Health, Indianapolis, IN) at 14 d intervals beginning 60 ± 3 DIM until dried off.

Treatments

Experiment 1. Lactating non-pregnant primiparous (n = 8) and multiparous (n = 31) Holstein cows at various DIM were synchronized using the Ovsynch protocol (Pursley et al.,

1995) with two PGF2α (25 mg Lutalyse, Zoetis Animal Health, New York, NY) injections (GnRH-7 d-PGF2α-1 d-PGF2α -32 h-GnRH). At the time of and 48 h after the second GnRH treatment of Ovsynch, transrectal ultrasonography (TUS) of the ovaries was performed to confirm ovulation which was defined as the disappearance of at least one follicle ≥10 mm and the presence of a putative corpus luteum (CL) on the same ovary. Six cows were excluded because they failed to ovulate after the GnRH treatment. Average (± SD) DIM and body condition score [BCS; scale 1 to 5 (Edmonson et al., 1989)] for the 6 primiparous and 27 multiparous cows that responded to the synchronization treatment and ovulated were 340 ± 244 d and 3.3 ± 0.7, respectively.

Seven days after induction of ovulation with the second GnRH treatment of Ovsynch, cows received two PGF2α treatments 12 h apart to induce luteal regression. Forty eight hours after the first PGF2α treatment (Day = 0), cows were stratified by parity (primiparous vs multiparous) and DIM and randomly assigned to one of five treatments: 1) 2 mL of saline solution IVG (SAL, n = 6), 2) 100 μg (2 mL) of Gonadorelin i.m. in the semimembranosus or semitendinosus muscle (G100-IM, n = 5), 3) 100 μg (2 mL) of Gonadorelin IVG (G100, n = 7), 4) 500 μg (10 mL) of Gonadorelin IVG (G500, n = 8), and 5) 1,000 μg (20 mL) of Gonadorelin IVG (G1000, n = 7).

Before application of the IVG treatments, the vulva and perineal area were washed and disinfected with chlorhexidine solution and dried off with paper towels. Thereafter, vulvar labia were manually opened by one technician while another technician inserted a uterine infusion catheter (44.5 cm long by 0.5 cm of external diameter) attached to a plastic syringe into the vaginal opening. The catheter was moved cranially until the cervix or vaginal fornix was reached. Once in the cranial portion of the vagina the catheter was pulled backwards 1 to 2 cm

and the volume of saline solution or GnRH analogue for the specific treatment was instilled. After treatment, cows remained standing for a minimum of 45 minutes to record urination activity and any significant amount of fluid backflow from the vagina.

On Day -2, Day 0 (< 30 min before treatment), and every 8 h for up to 48 h after treatment, the position and size of all antral follicles ≥ 8 mm in diameter was determined by TUS and recorded for subsequent determination of the occurrence and timing of ovulation after treatment. Timing of ovulation was defined as the mid-point between the last examination in which the putative ovulatory follicle was visualized and the first examination in which the putative ovulatory follicle was not visualized through TUS.

Experiment 2. Lactating nonpregnant multiparous (parity 2 to 5) Holstein cows (n = 59) were enrolled and received the synchronization of ovulation protocol described for Experiment 1 except that a different PGF2α product was used (Estrumate, Merck Animal Health, Summit, NJ). Average (± SD) DIM, body weight (BW), and BCS for the 48 cows that responded to the synchronization treatment and ovulated were 83 ± 48 d, 721 ± 54 kg and 3.3 ± 0.5 respectively.

At the time of treatment (Day = 0; 48 h after first PGF2α treatment following the Ovsynch protocol) cows were randomly assigned to receive one of six treatments: 1) 2 mL of saline solution IVG (SAL, n = 8), 2) 100 μg (2 mL) of Gonadorelin i.m. in the semimembranosus or semitendinosus muscle (G100-IM, n = 8), 3) 1,000 μg (20 mL) of Gonadorelin IVG (G1000, n = 8), 4) 1,000 μg of Gonadorelin plus 10% citric acid (20 mL) IVG (G1000CA , n = 8), 5) 80 μg (20 mL) of Buserelin IVG (B80, n = 8), and 6) 80 μg of Buserelin plus 10% citric acid (20 mL) IVG (B80CA, n = 8). The dose selected for Buserelin treatment was equivalent to 10 times the recommended dose to induce ovulation in cattle. Treatments were performed following the same procedures described for Experiment 1.

To determine if the IVG treatments caused irritation of the vaginal mucosa, on Day -1 and 8 h after treatment, the vaginal mucosa was visually inspected using a speculum and a source of light.

GnRH Analogue Solutions Used for Treatments

In Experiment 1, the GnRH analogue Gonadorelin (50 µg/mL of Gonadorelin diacetate tetrahydrate, Cystorelin, Merial LLC, Duluth, GA) was used for the i.m. and IVG treatments. In Experiment 2, Gonadorelin was used for the i.m. treatment and either Gonadorelin or Buserelin were used for the IVG treatments. The same commercially available Gonadorelin product was used in Experiment 2, whereas Buserelin solution (no commercially available product for cattle in the U.S.) was prepared in our laboratory using Buserelin acetate salt (Sigma-Aldrich, Saint Louis, MO). The solution was prepared by adding Buserelin and benzyl alcohol (Sigma-Aldrich, Saint Louis, MO) to reach a final concentration of 4 µg/mL of Buserelin and 9 mg/mL of benzyl alcohol. Thereafter, the pH of the solution was adjusted using NaOH and HCl to reach a mean pH of 6.7. Solutions of Gonadorelin and Buserelin with citric acid were prepared by adding citric acid (C0759-500G, Sigma -Aldrich, Saint Louis, MO) 10% m/v. The final pH was balanced using NaOH and HCl to reach a mean of 4.01 because an acid pH has been shown to increase GnRH absorption by the vagina in rats (Okada et al., 1983). Citric acid was chosen as absorption enhancer because it chelates calcium which has been shown to loosen intercellular tight junctions, thus facilitating intercellular transport (Cho et al., 1989). In addition, there was evidence that citric acid increased absorption and vaginal permeability of the GnRH analogue Leuprolide in rats (Okada et al., 1982, 1983).

Blood Sample Collection

On Day -7, -2, and 0 (time of treatment) blood samples were collected from the coccygeal vein or artery using evacuated tubes containing sodium heparin (Vacutainer, BD, Franklin Lakes, NJ) for P4 and estradiol (E2; Day 0 only) analysis to confirm response to the synchronization of ovulation protocol, and circulating concentrations of P4 and E2 before treatment.

To determine circulating LH concentrations, samples (4 mL) were obtained from the jugular vein using a syringe attached to an indwelling jugular catheter. Samples were collected every 15 min from -15 min to 4 h after treatment and every 30 min from 4 to 6 h after treatment. Immediately after collection, samples were poured into tubes containing sodium heparin (10 µl of 6.7 IU/mL of heparin solution) and placed in crushed ice until centrifugation (within 2 h of collection) for 20 min at 2,000 x g in a refrigerated centrifuge set at 4°C. After centrifugation, plasma samples were harvested and stored at -20°C until assayed.

Jugular catheters were placed 24 h before treatment. Briefly, cows were restrained in an individual cow chute and a halter was used to immobilize the head and neck. The area of the neck where the catheter was inserted was shaved using clippers followed by a first wash with povidone iodine solution and a second wash with 70% ethanol. A 13 gauge x 13 cm long polyurethane catheter over needle (Mila International, France, KY) was inserted into the jugular vein, the needle was removed and the catheter secured in place using a temporary suture. A 53 cm extension tubing set (Baxter healthcare corporation, Deerfield, IL) was attached to the catheter to facilitate sample collection. Sterile saline solution containing 20 IU/mL of heparin was used to prevent clotting and maintain the catheter permeable until sampling began.

Determination of Circulating Concentrations of Progesterone

Progesterone concentrations were estimated in duplicate using a commercial solid-phase, no-extraction radio immunoassay (RIA; Experiment 1: Coat-a-count; Diagnostic Products Corp., Los Angeles, CA; Experiment 2: ImmuChem Coated Tube, MP Biomedicals, Costa Mesa, CA). To assess the precision of the assay, control samples with a high (7.5 and 6.0 ng/mL for experiments1 and 2 respectively) and low (0.5 and 0.3 ng/mL for experiments 1 and 2, respectively) concentrations of P4 were assayed. Average sensitivity for the P4 assay was 0.03 ng/mL for Experiment 1 and 0.1 ng/mL for Experiment 2. The intra-assay CV for the high-concentration sample was 10.6% in Experiment 1 and 7.9% in Experiment 2. The CV for the low concentration sample was 4.9% in Experiment 1 and 13.9% in Experiment 2.

Determination of Circulating Concentrations of Estradiol

Circulating concentrations of estradiol were only determined for samples from Experiment 1 because the assay used for Experiment 1 was no longer available and no other assay with adequate sensitivity was available to run samples from Experiment 2 (collected at a later time).

Samples were assayed in duplicate for estimation of E2 concentrations using a double antibody RIA after benzene:toluene extraction. Analysis was performed as described in Beam and Butler (1997) using a commercially available kit (MaiaZen Estradiol R-FA-120, Zen Tech SA, Belgium). Average sensitivity for the assay was 0.3 pg/mL. A quality control sample (6.5 pg/mL) was added in quadruplicate (2 x 100 μL and 2 x 200 μL) at the beginning and end of each assay to assess the precision of the assay. Intra-assay CV was 16%.

Determination of Circulating Concentrations of Luteinizing Hormone

Concentrations of LH in plasma samples (200 µL) from both experiments were determined using two different double antibody RIA. In Experiment 1, assays (n = 2) were conducted as in Price et al. (1987) with the modifications described in Butler et al. (2004). Samples containing known amounts of LH (2.5 ng/mL) were included at three different dilutions at the beginning and end of each assay. These control samples showed parallelism with the standard curve. Mean limit of detection, defined as 95% binding, was 0.2 ng/mL. Intra and inter-assay CV were 10% and 8%, respectively.

For LH assays used in Experiment 2 (n = 4), bovine LH (bLH AFP-11118B; National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) was used to make iodinated tracer and standards. Rabbit anti-bovine LH antiserum was used as first antibody (AFP-192279; National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA). On Day 1, buffer, sample (200 µL) or standards, tracer, and first antibody were added to assay tubes and incubated at room temperature for 24 h. On Day 2, the secondary antibody was added and incubated at room temperature for 24 h. Thereafter, tubes were incubated at 4°C for an additional 24 h. Samples containing high (4.5 ng/mL) and low (2.5 ng/mL) LH concentrations were included at the beginning and end of each assay at three different dilutions. Control samples showed parallelism with the standard curve. Mean limit of detection, defined as 95% binding, was 0.7 ng/mL for the four assays. The intra-assay CV was 9% and 13% and the inter-assay CV was 9% and 26% for the high and low control sample, respectively.

In both systems the standard curve range was 0.1 to 4 ng which is equivalent to 0.5 to 20 ng/mL.

Statistical Analysis

Data from Experiment 1 and 2 were analyzed using the same methodology unless stated otherwise.

A surge of LH after treatment (Time 0) was defined as an increase in mean LH concentrations from basal levels (i.e., mean for -15 and 0 min after treatment) equal or greater to the mean increase to maximum concentration in ng/mL of LH observed for cows in the G100-IM treatment minus two SD. This calculation was conducted separately for Experiments 1 and 2. Area under the curve (AUC) for LH concentration from -15 to 360 min after treatment was approximated through the trapezoidal method as described in Giordano et al. (2012a). For continuous variables (concentrations of P4, E2, and LH, AUC, maximum LH concentration, time to maximum LH concentration, time to ovulation, DIM, BCS, and BW) normality of residuals and homoscedasticity of variance were verified using graphical methods (Q-Q plot and conditional studentized residual plot) generated with the residual option of the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). In both experiments mean LH concentrations had a non-normal distribution and studentized residuals showed heteroscedasticity of variance. Because none of the data transformation methods used (natural logarithm, square root, and inverse transformation) eliminated the heteroscedasticity of variance, the natural logarithm of LH concentration was used for Experiment 1 and the inverse of LH concentration was used for Experiment 2. The final models were selected based on the lowest value for the AIC and BIC (Littell et al., 2006). Transformed mean LH concentration values were analyzed using ANOVA with repeated measures using the MIXED procedure of SAS including the Satterthwaite approximation. In order to adjust for varying intervals in blood

sample collection, a spatial power covariance structure was used. Treatment, time, and the treatment by time interaction were included as fixed effects. Cow nested within treatment was included as a random effect in the models.

Data for P4 concentration at Time 0, AUC, maximum LH concentration, and time to maximum LH concentration had a non-normal distribution and were transformed to natural logarithm for analysis. The aforementioned variables and BCS, DIM, BW, size of the largest follicle at the time of treatment and E2 concentration at Time 0 were analyzed using ANOVA with the MIXED procedure of SAS with a model that included treatment as fixed effect. The Tukey post-hoc mean separation test was used to determine differences between LSM. Due to lack of normality and heteroscedasticity of the data, time to ovulation was analyzed using the Kruskal-Wallis test with the NPAR1WAY procedure of SAS, using a model that included treatment as the classification variable.

Binomial outcomes (proportion of cows with a surge of LH and ovulatory response after treatment) were analyzed using Fisher's exact test with the FREQ procedure of SAS because some treatments had either 0% or 100% of the cows with a positive outcome. Mean separation analysis was not conducted for binomial outcomes because there was an insufficient number of observations for a meaningful comparison.

All values for continuous variables are presented as arithmetical means and SE generated with the MEANS procedure of SAS. All proportions were generated using the FREQ procedure of SAS. All explanatory variables included as fixed effects in models were considered significant if $P < 0.05$, whereas $0.05 \leq P \leq 0.10$ were considered a tendency.

RESULTS

Experiment 1

No signs of discomfort were observed after treatment in any of the cows. No urination or backflow of fluid from the vagina was observed for up to 45 min after treatment. One cow from the G100 treatment was removed from the data analysis because it presented elevated P4 (2.1 ng/mL) concentration at the time of treatment. No statistical difference was observed between treatments for DIM ($P = 0.94$) and BCS ($P = 0.46$).

Size of the largest follicle at the time of treatment. Size of the largest follicle was not different ($P = 0.63$) among treatments (SAL = 17.5 ± 0.6 mm, G100-IM = 16.1 ± 1.9 mm, G100 = 17.8 ± 0.3 mm, G500 = 18.3 ± 0.7 mm, and G1000 = 19.1 ± 2.0 mm).

Circulating Concentrations of Progesterone and Estradiol at Time 0. Mean P4 and E2 concentrations at the time of treatment were not different ($P > 0.1$) among treatments (Table 1). All cows had P4 concentrations below 0.5 ng/mL at Time 0 except one cow from the SAL and one cow from the G100 treatment which had P4 concentrations of 0.8 and 0.9 ng/mL, respectively.

***Circulating Concentrations of LH*.** Treatment affected ($P < 0.001$) AUC for LH. Cows in the G100-IM treatment had the greatest AUC whereas cows in the G1000 treatment had greater AUC than cows in the SAL and G500 treatments (Table 1).

There was an effect of treatment ($P < 0.001$), time ($P < 0.001$), and treatment by time interaction ($P < 0.001$) for mean circulating LH concentrations (Figure 3.2).

Treatment affected ($P < 0.001$) the maximum LH concentration reached during the sampling period. Cows in the G100-IM treatment had the greatest maximum LH concentration whereas cows in the G1000 treatment had greater maximum LH concentration than cows in the SAL and G500 treatments (Table 1). Treatment affected ($P < 0.001$) the proportion of cows with

a surge of LH within 6 h of treatment. None of the cows in the IVG treatments had an increase in LH concentrations consistent with a surge of LH as defined for this experiment (Table 1). Time to maximum LH concentration was not compared between treatments because only cows in the G100-IM treatment had a surge of LH.

Ovulatory Response to Treatment. Although this study was not designed to validate treatment effects on binomial outcomes such as ovulatory response, this outcome was recorded and analyzed. Treatment did not affect the proportion of cows that ovulated within 48 h of treatment ($P = 0.28$) or mean time to ovulation ($P = 0.17$; Table 1).

Experiment 2

No signs of discomfort or alteration of the vaginal mucosa detectable through visual inspection were observed after treatment in any of the cows. One cow from to the G1000 treatment was removed from the data analysis because LH concentration at 15 min before treatment were 889% greater than the mean of the other cows in the G1000 treatment (6.67 vs 0.75 ng/mL). No statistical difference was observed between treatments for DIM ($P = 0.72$), BW ($P = 0.85$), and BCS ($P = 0.98$).

Circulating Concentrations of Progesterone at Time of Treatment. No statistical analysis was performed for P4 concentration at Time 0 because mean P4 concentrations were at or below the assay detection limit (0.1 ng/mL) in all treatments (Table 2). None of the cows had P4 concentrations above 0.2 ng/mL.

Size of the largest follicle at the time of treatment. Size of the largest follicle was not different ($P = 0.86$) among treatments (SAL = 20.5 ± 1.5 mm, G100-IM = 18.5 ± 0.5 mm, G1000

= 18.6 ± 0.9 mm, G1000CA = 19.1 ± 1.0 mm, B80 = 18.9 ± 0.6 mm, and B80CA = 18.9 ± 1.9 mm).

Circulating LH Concentrations. Treatment affected ($P < 0.001$) the AUC for LH concentrations. The AUC was greater for cows in the G100-IM, G1000CA, and B80CA treatments than for the other IVG treatments (Table 2).

Mean circulating LH concentrations during the experimental period were affected by treatment ($P < 0.001$), time ($P < 0.001$), and the treatment by time interaction ($P < 0.001$; Figure 3.3).

Treatment affected ($P < 0.001$) the proportion of cows that had a surge of LH within the sampling period (Table 3.2). Treatment affected ($P < 0.001$) maximum LH concentration during the experimental period because it was greater for the G100-IM, G1000CA, and B80CA treatments than for the rest of the IVG treatments (Table 3.2).

Because no cows in the SAL treatment and only one cow in the G1000 and B80 treatments had a surge of LH, these treatments were not included in the analysis of time to maximum LH concentration. Only cows in the G100-IM, G1000CA, and B80CA treatments with an evident LH surge were included in the analysis for time to maximum LH concentration. Treatment affected ($P < 0.001$) time to maximum LH concentration because the G100-IM treatment had the shortest interval from treatment to maximum LH concentration and the G1000CA had shorter time to maximum LH concentration than the B80CA treatment (Table 3.2).

Ovulatory Response to Treatment. Although this study was not designed to validly evaluate treatment effects on binomial outcomes such as ovulatory response, this outcome was

recorded and analyzed. Treatment did not affect ($P = 0.11$) the proportion of cows that ovulated within 48 h of treatment and tended ($P = 0.07$) to affect mean time to ovulation (Table 3.2).

Table 3.1. Steroid hormone concentration (progesterone and estradiol) at the time of treatment, LH secretion dynamics, and ovulation after treatment in Experiment 1.

Treatment[1]	P4[2] Time 0 (ng/mL)	E2[3] Time 0 (pg/mL)	AUC[4]	Maximum LH (ng/mL)	LH surge (%; n)	Time to maximum LH (min)[5]	Ovulation[6] (%; n)	Time to ovulation[7] (h)
SAL (n = 6)	0.3 ± 0.1	4.2 ± 0.9	242 ± 63^{c}	1.1 ± 0.2 ^{c}	0 (0)	-	67 (4)	34 ± 5
G100-IM (n = 5)	0.1 ± 0.1	4.6 ± 0.8	1,149 ± 69^{a}	6.9 ± 0.4 ^{a}	100 (5)	81 ± 4	100 (5)	28
G100 (n = 6)[8]	0.2 ± 0.1	4.2 ± 0.4	286 ± 36^{bc}	1.4 ± 0.2^{bc}	0 (0)	-	50 (3)	38 ± 3
G500 (n = 8)	0.2 ± 0.1	4.7 ± 0.8	247 ± 55^{c}	1.2 ± 0.1^{c}	0 (0)	-	75 (6)	36 ± 5
G1000 (n = 7)	0.1 ± 0.1	5.4 ± 1.0	546 ± 58^{b}	2.8 ± 0.6^{b}	0 (0)	-	86 (6)	37 ± 3
P-value	0.25	0.82	< 0.001	< 0.001	< 0.001	-	0.45	0.17

[1]Forty eight hours after the first of two PGF2α treatments given 12 h apart, cows were randomly assigned to receive the following treatments: 2 ml of saline intravaginal (SAL), 100 µg of Gonadorelin i.m. (G100-IM), 100 µg of Gonadorelin IVG (G100), 500 µg of Gonadorelin IVG (G500), or 1,000 µg of Gonadorelin IVG (G1000). Blood samples were collected every 15 min from -15 min to 4 h after treatment and every 30 min from 4 h until 6 h after treatment.

[2]P4 = progesterone

[3]E2 = estradiol

[4]AUC = area under the curve

[5]Only for cows that had a surge of LH.

[6]Transrectal ultrasonography of the ovaries was performed every 8 h from Time 0 until 48 h after treatment to detect ovulation.

[7]Standard error of the mean not reported because it was 0.

[8]One cow was removed from the data analysis because it presented elevated P4 (2.1 ng/mL) concentration at the time of treatment.

Different superscripts within a column differ statistically based on Tukey's mean separation test.

Table 3.2. Concentration of progesterone at the time of treatment, LH secretion dynamics, and ovulation after treatment in Experiment 2.

Treatment[1]	P4[2] Time 0 (ng/mL)	AUC[3]	LH surge (%; n)	Maximum LH (ng/mL)	Time to maximum LH[4] (min)	Ovulation[5] (%; n)	Time to Ovulation[6] (h)
SAL (n = 8)	0.1	356 ± 36[b]	0 (0)	1.4 ± 0.4[b]	-	50 (4)	38 ± 6
G100-IM (n = 8)	0.1	1,781 ± 191[a]	100 (8)	9.7 ± 0.9[a]	101 ± 8[c]	100 (8)	28 ± 2
G1000 (n = 7)[7]	0.1	615 ± 200[b]	14 (1)	3.1 ± 1.3[b]	-	57 (4)	36 ± 5
G1000CA (n = 8)	0.1	1,977 ± 309[a]	88 (7)	9.8 ± 1.5[a]	145 ± 5[b]	88 (7)	28
B80 (n = 8)	0.1	466 ± 143[b]	13 (1)	1.7 ± 0.6[b]	-	88 (7)	37 ± 5
B80CA (n = 8)	0.1	1,946 ± 278[a]	100 (8)	9.3 ± 1.5[a]	185 ± 10[a]	88 (7)	28
P-value	-	< 0.001	< 0.001	< 0.001	< 0.001	0.11	0.07

[1]Forty eight hours after the first of two PGF2α treatments given 12 h apart, cows were randomly assigned to receive the following treatments: 2 ml of saline intravaginal (SAL), 100 µg of Gonadorelin i.m. (G100-IM), 1,000 µg of Gonadorelin IVG (G1000), 1,000 µg of Gonadorelin plus 10% citric acid IVG (G1000CA), 80 µg of Buserelin IVG (B80), 80 µg of Buserelin plus 10% citric acid IVG (B80CA). Blood samples were collected every 15 min from -15 min to 4 h after treatment and every 30 min from 4 h until 6 h after treatment.

[2]P4 = progesterone

[3]AUC = area under the curve

[4]Only for cows with a surge of LH in the G100-IM, G100CA, and B80CA treatments.

[5]Transrectal ultrasonography of the ovaries was performed every 8 h from Time 0 until 48 h after treatment to detect ovulation.

[6]Standard error of the mean not reported because it was 0.

[7]One cow was removed from the data analysis because LH concentration at 15 min before treatment were 889% greater than the mean of the other cows in the treatment (6.67 vs 0.75 ng/mL).

Different superscripts within a column differ statistically based on Tukey's mean separation test.

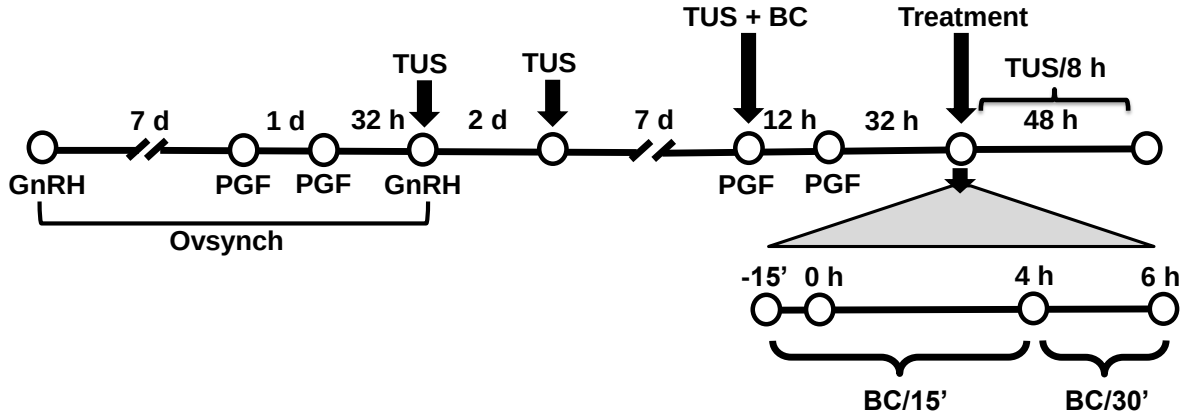

Figure 3.1. Graphical depiction of experimental procedures for Experiments 1 and 2. Cows were synchronized using a modified Ovsynch protocol with two PGF2α injections (GnRH-7 d-PGF2α -1 d-PGF2α -32 h-GnRH). Cows with at least one corpus luteum ≥15 mm and one follicle ≥15 mm 7 d after the second GnRH injection of Ovsynch remained in the study to receive the treatments. In Experiment 1, cows (n = 32) stratified by parity received: 2 mL of saline solution intravaginal (IVG; SAL, n = 6), an i.m. injection of 100 µg of Gonadorelin (G100-IM, n = 5), 100 µg of Gonadorelin IVG (G100, n = 6), 500 µg of Gonadorelin IVG (G500, n = 8), and 1,000 µg of Gonadorelin IVG (G1000, n = 7). In Experiment 2, cows (n = 47) stratified by parity received: 2 mL of saline solution IVG (SAL, n = 8), an i.m. injection of 100 µg of Gonadorelin (G100-IM, n = 8), 1,000 µg of Gonadorelin IVG (G1000, n = 7), 1,000 µg of Gonadorelin plus 10% citric acid IVG (G1000CA, n = 8), 80 µg of Buserelin IVG (B80, n = 8,) and 80 µg of Buserelin plus 10% citric acid IVG (B80CA, n = 8). Blood was collected every 15 min from 15 min pretreatment up to 4 h, and then every 30 min up to 6 h after treatment. PGF = PGF2α, TUS = transrectal ultrasonography, BC = blood collection.

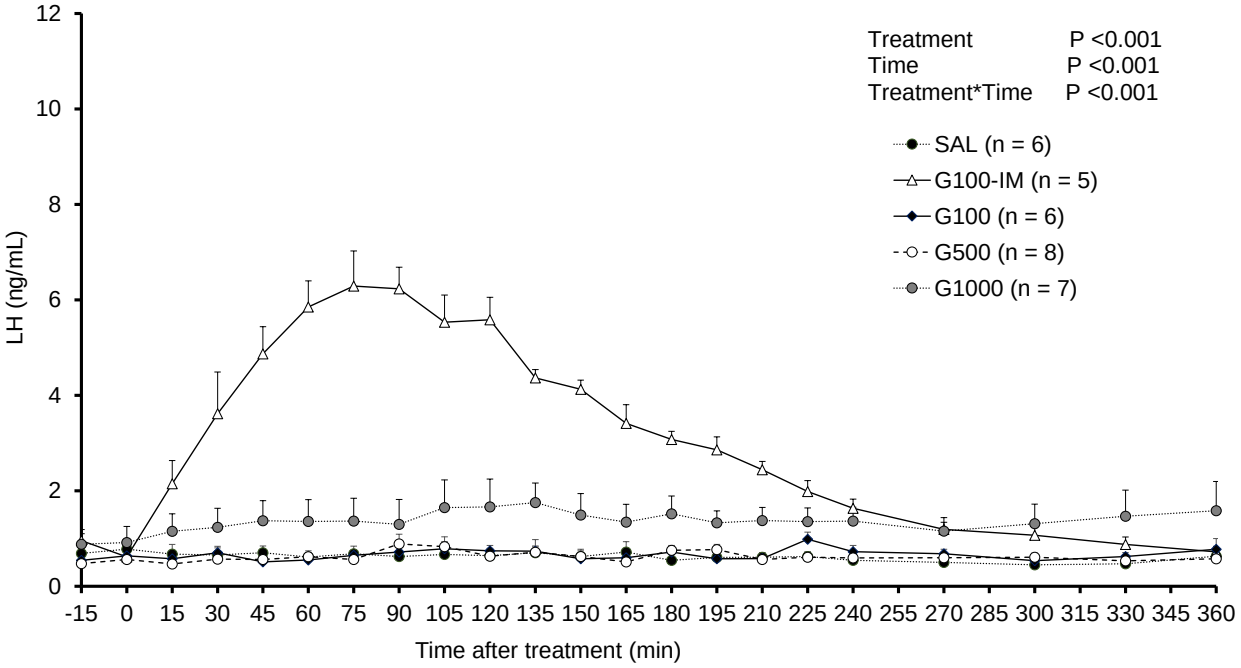

Figure 3.2. Mean circulating LH concentrations from -15 min to 6 h after treatment for Experiment 1. Seven days after the second GnRH injection of a modified Ovsynch protocol, cows received two PGF2α treatments 12 h apart. Forty-eight hours after the first PGF2α treatment, cows were randomly assigned to the following treatments: SAL = 2 ml of saline intravaginal (IVG), G100-IM = 100 µg of Gonadorelin i.m., G100 = 100 µg of Gonadorelin IVG, G500 = 500 µg of Gonadorelin IVG, G1000 = 1,000 µg of Gonadorelin IVG. Blood was collected every 15 min from 15 min pretreatment up to 4 h, and then every 30 min up to 6 h after treatment. There was an effect of treatment ($P < 0.001$), time ($P < 0.001$), and an interaction between treatment and time ($P < 0.001$) on circulating concentration of LH.

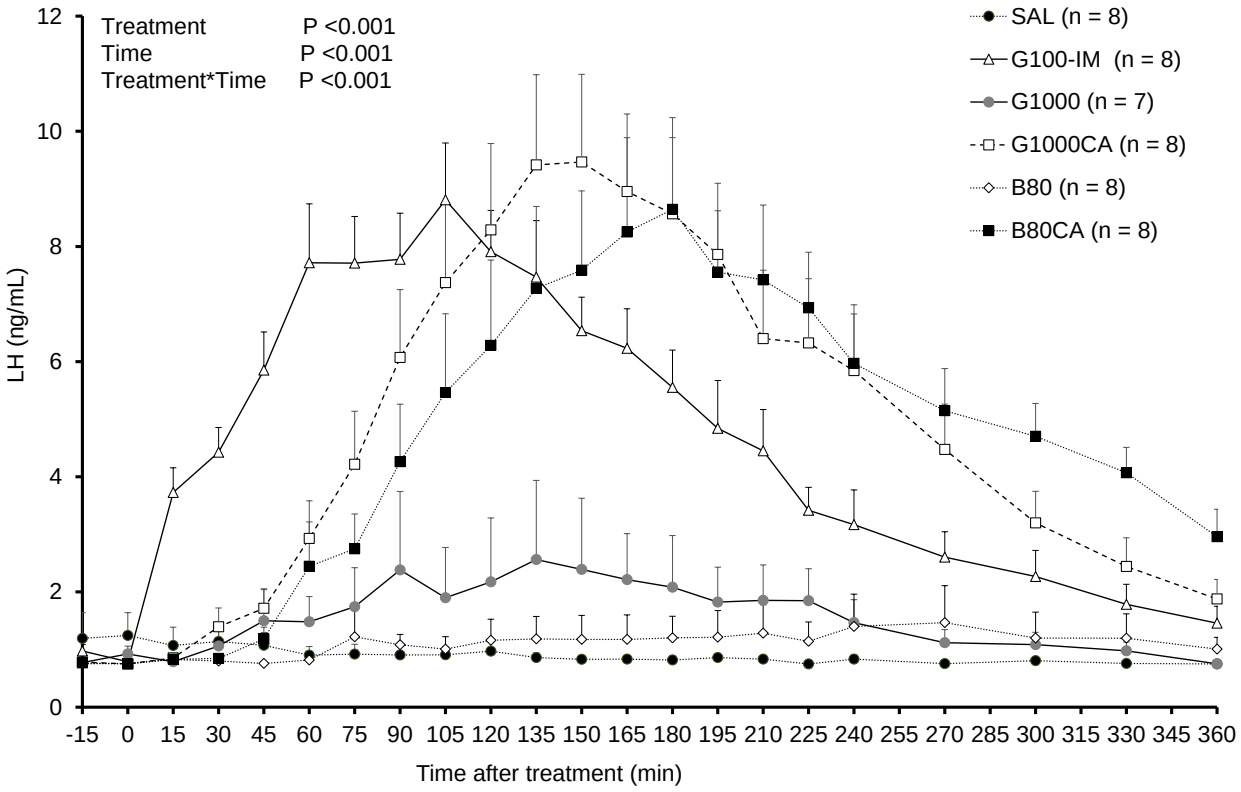

Figure 3.3. Mean circulating concentration of LH from -15 min to 6 h after treatment for Experiment 2. Seven days after the second GnRH injection of a modified Ovsynch protocol, cows received two PGF2α treatments 12 h apart. Forty eight hours after the first PGF2α treatment, cows were randomly assigned to the following treatments: SAL = 2 ml of saline intravaginal (IVG), G100-IM = 100 µg Gonadorelin i.m., G1000 = 1,000 µg Gonadorelin IVG, G1000CA = 1,000 µg Gonadorelin plus 10% citric acid IVG, B80 = 80 µg Buserelin IVG, B80CA = 80 µg Buserelin plus 10% citric acid IVG. Blood was collected every 15 min from 15 min pretreatment up to 4 h, and then every 30 min up to 6 h after treatment. There was an effect of treatment ($P < 0.001$), time ($P < 0.001$), and an interaction between treatment and time ($P < 0.001$) on circulating concentration of LH.

DISCUSSION

The main objective of this research was to test the feasibility of using intravaginal instillation of GnRH analogues for inducing a surge of LH in cattle. We aimed to induce a surge of LH similar to that induced after i.m. injection of the labeled dose of GnRH (100 µg of Gonadorelin) for synchronization of ovulation in cattle. In Experiment 1 three different doses of Gonadorelin equivalent to 1, 5, and 10 times the labeled i.m. dose of Gonadorelin were instilled in the vagina of lactating dairy cows. Results from this experiment indicated that IVG instillation of Gonadorelin did not elicit a surge of LH of the same magnitude and timing observed after i.m. injection of a 100 µg dose of Gonadorelin. The lack of response (i.e., no difference from basal LH concentrations up to 6 h after treatment) to the smaller doses of Gonadorelin suggests that either no Gonadorelin or less than the minimum amount required to cause a substantial increment in circulating LH concentrations reached the pituitary gland. Conversely, the low magnitude but detectable increment in circulating LH concentrations observed for cows in the G1000 treatment suggests that at least part of the Gonadorelin was absorbed and reached the pituitary gland. Although we cannot rule out the possibility that a surge of LH occurred after sampling concluded, the rate of absorption and/or the amount absorbed was inadequate to elicit release of large amounts of LH in a surge-like pattern up to the end of the sampling period.

In two experiments Bas et al. (2012; 2014) compared intrauterine administration of 100 or 200 µg of Gonadorelin with i.m. injection of 100 µg of Gonadorelin in lactating dairy cows. In both cases intrauterine administration of Gonadorelin failed to mimic the LH surge elicited by i.m. administration. The LH surge observed was of low magnitude and mean LH concentrations did not differ from that of cows that received intrauterine saline. Collectively, the results of our first experiment and others (Bas et al., 2012, 2014) suggest that absorption of Gonadorelin

through intact vaginal or uterine epithelium of lactating dairy cows is limited which, in turn, leads to insufficient absorption of Gonadorelin to induce a surge of LH of similar magnitude and timing than that observed after i.m. injection.

By design the endocrine environment at the time of treatment in cows from Experiment 1 was optimal for LH secretion (Giordano et al., 2012a; Lima et al., 2013; Pulley et al., 2015). All cows had sub luteal (< 1 ng/mL) concentrations of P4 and similar circulating concentrations of E2 than observed during estrus (Lucy and Stevenson, 1986) when the endogenous LH surge responsible for triggering ovulation is elicited by E2 (Moenter et al., 1990). It is also well known that cattle which receive i.m. GnRH when circulating concentrations of P4 are low (< 1 ng/mL) and circulating concentrations of E2 are high, have greater LH release than when circulating concentrations of P4 are high and E2 concentrations are low (Giordano et al., 2012a; Pulley et al., 2015; Stevenson and Pulley, 2016). In spite of the adequate endocrine environment at the time of treatment, IVG instillation of Gonadorelin either did not elicit LH release or the amount released was less than that observed for cows in the i.m. treatment. Only cows in the G1000 treatment had a detectable increase in circulating LH (AUC and maximum LH concentration). Collectively, these observations support the notion that vaginal absorption was substantially less than after i.m. injection and the greater amount of Gonadorelin provided to cows in the G1000 treatment only partially offset poor absorption. Because it is unlikely that the pharmacokinetics of a GnRH analogue is affected after it reaches the blood stream when given through different routes of administration, we speculated that major reasons for the poor response to IVG treatment with Gonadorelin were limited passage through the mucosa, degradation before absorption, or both. Reduced passage (or absorption) may have been the result of the physical barrier created by the inter-cellular tight junctions of the vaginal epithelium. Degradation of

GnRH analogues is possible because it has been shown that the vaginal mucus from various species (i.e., human, sheep, rat and rabbit) contains proteolytic enzymes which may be able to degrade GnRH analogues (Han et al., 1995; Acartürk et al., 2001). Although we cannot completely rule out the possibility of a poor response due to backflow of GnRH, we did not observe backflow from the vagina for up 45 min after treatment.

Thus, our objective for Experiment 2 was to increase absorption of GnRH analogues through the vaginal mucosa by adding citric acid to the GnRH analogue solutions. Increasing absorption of GnRH analogues was expected to help induce a surge of LH of at least similar magnitude and timing than that observed after i.m. administration of Gonadorelin. In support of our hypothesis, results from this experiment provided evidence that including citric acid as an absorption enhancer was a feasible strategy to induce a surge of LH after IVG instillation of GnRH analogues in lactating dairy cows. The addition of citric acid to Gonadorelin and Buserelin facilitated the induction of a surge of LH in 88% and 100% of cows in the G1000CA and B80CA treatments, respectively. Based on the AUC and maximum LH concentration, the LH surge was of similar magnitude than that observed for cows that received Gonadorelin i.m. These findings are in agreement with experiments performed in rats where vaginal absorption of the GnRH analogue Leuprolide increased after addition of citric acid (Okada 1982, 1983). Addition of this acid facilitates intercellular transport of peptides as it loosens intercellular tight junctions by chelating Ca^{2+} (Cho et al., 1989). In addition, the reduction in pH may cause alterations to membrane and peptide charges that facilitate absorption of hydrophilic molecules (Okada et al., 1983).

Addition of citric acid benefited absorption of the GnRH analogues; however, the similar magnitude of the LH surge in cows that received 100 µg of Gonadorelin i.m. and cows in the

G1000CA and B80CA treatments suggested that IVG absorption of Gonadorelin and Buserelin was less efficient than after an i.m. injection of Gonadorelin. The treatments that included citric acid also had a clear shift in the timing of LH release which resulted in greater intervals from treatment to maximum concentration of LH. This interval was at least 30 to 110 min longer than for cows which received Gonadorelin i.m. in ours and other experiments (Giordano et al., 2012a; Picard-Hagen et al., 2015; Pulley et al., 2015). This delay may have been the consequence of the additional time required for citric acid to exert its action on the vaginal epithelium. It also suggests that the greatest limitation to absorption of GnRH analogues was the inability to pass through the mucosa rather than degradation by proteolytic enzymes present in the vaginal mucus.

We also explored the possibility that the potent GnRH analog Buserelin would elicit greater LH release than Gonadorelin. Our reasoning was that because of its greater potency [i.e., ~50 times more potent; (Chenault et al., 1990; Picard-Hagen et al., 2015)], Buserelin would be less dependent on absorption of large quantities than Gonadorelin. We did not observe a substantial difference in LH concentrations between treatments that received Gonadorelin or Buserelin with or without citric acid. In fact, the greatest difference in LH profiles between the two analogues was the longer interval from treatment to maximum LH concentration in the B80CA treatment. Although this remains speculative, our observations suggest that vaginal absorption of Buserelin might have been less efficient than that of Gonadorelin. Modifications to the molecular structure of Buserelin to increase its potency (Millar, 2005) may have been responsible for the reduced absorption and/or a different response to the reduction in pH.

Our current experiments were not designed to and were underpowered to evaluate ovulatory response to treatment. Nevertheless, the observed incidence and timing of ovulation were intriguing. As expected all cows with a surge of LH ovulated within 48 h of treatment

except for one cow in the B80CA treatment (data not shown). Interestingly, 64% of cows that did not have a surge of LH (data combined from Experiments 1 and 2) during the sampling period ovulated within 48 h of treatment. Then, it is reasonable to speculate that we could have detected more cows with a surge of LH if the sampling period was extended. Whether these LH surges would have been spontaneous or induced by the IVG treatment with the GnRH analogues would have been difficult to determine. The fact that 57% of the cows in the SAL treatment ovulated suggests that at least in some cows a spontaneous and not an induced LH surge occurred after the end of the sampling period. Moreover, although it is rather unlikely, we cannot rule out the possibility that some of these cows had an endogenous LH surge before the time of treatment.

As expected, cows that had a surge of LH during the sampling period tended to ovulate earlier and timing of ovulation for cows that ovulated in the G100-IM, G1000CA, and B80CA was within the expected time frame after GnRH treatment (Pursley et al., 1995; Rantala et al., 2009; Giordano et al., 2012a). In spite of the fact that cows in the G1000CA and B80CA treatments had a longer interval until LH concentrations reached their peak, mean time to ovulation was the same in all treatments. Therefore, the delay in reaching maximum concentration of LH observed in our experiment may not substantially affect timing of ovulation in cows. Additional research with more cows is necessary to determine if IVG instillation of GnRH analogues leads to differences in ovulatory response and time to ovulation as compared with i.m. injection.

CONCLUSION

Based on results from Experiment 1, we concluded that Gonadorelin absorption through intact vaginal epithelium was insufficient to elicit a surge of LH of similar magnitude and timing

than that observed after i.m. injection of Gonadorelin. Based on the results of Experiment 2, we concluded that citric acid can enhance absorption of Gonadorelin or Buserelin through the vaginal mucosa. A dose of Gonadorelin and Buserelin 10 times greater than the regularly used i.m. dose of these GnRH analogues plus 10% citric acid, induced a surge of LH of similar magnitude than that induced after i.m. injection of a 100 μg dose of Gonadorelin. More research is needed to elucidate differences in pharmacokinetics of GnRH and its analogues after IVG administration in order to establish the most appropriate dose, volume, and vehicle to effectively induce a surge of LH capable of triggering ovulation in cattle.

Results from the current experiments and previous research on IVG instillation of PGF2α in lactating dairy cows (Wijma et al., 2016) support the potential use of the vagina as a route of administration for reproductive hormones used in synchronization of estrus and/or ovulation protocols. Demonstrating the feasibility of IVG hormone administration is a necessary first step for facilitating the development of automated IVG hormone delivery devices which, in turn, might help improve the value of synchronization of estrus and ovulation protocols for cattle operations.

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REFERENCES

Acartürk, F., Z. I. Parlatan, and Ö. F. Saracoĝlu. 2001. Comparison of vaginal aminopeptidase enzymatic activities in various animals and in humans. Journal of Pharmacy and Pharmacology 53:1499-1504.

Bas, S., M. Maquivar, M. C. da Silva, M. Day, M. Daglio, S. Harguindeguy, M. Titler, and G. M. Schuenemann. 2014. Effect of intrauterine administration of gonadotropin releasing hormone with glycerol on serum LH concentrations in lactating dairy cows. Animal reproduction science 145:15-22.

Bas, S., C. Pinto, M. Day, and G. Schuenemann. 2012. Effect of intrauterine administration of gonadotropin releasing hormone on serum LH concentrations in lactating dairy cows. Theriogenology 78:1390-1397.

Beam, S. W. and W. Butler. 1997. Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. Biology of reproduction 56:133-142.

Butler, S. T., S. Pelton, and W. Butler. 2004. Insulin increases 17β-estradiol production by the dominant follicle of the first postpartum follicle wave in dairy cows. Reproduction 127:537-545.

Chebel, R., J. Santos, R. Cerri, H. Rutigliano, and R. Bruno. 2006. Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. Journal of dairy science 89:4205-4219.

Chenault, J., D. Kratzer, R. Rzepkowski, and M. Goodwin. 1990. LH and FSH response of Holstein heifers to fertirelin acetate, Gonadorelin and Buserelin. Theriogenology 34:81-98.

Cho, M., J. Scieszka, and P. Burton. 1989. Citric acid as an adjuvant for transepithelial transport. International journal of pharmaceutics 52:79-81.

Cross, P. S., R. Künnemeyer, C. R. Bunt, D. A. Carnegie, and M. J. Rathbone. 2004. Control, communication and monitoring of intravaginal drug delivery in dairy cows. International journal of pharmaceutics 282:35-44.

Edmonson, A., I. Lean, L. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. Journal of dairy science 72:68-78.

Fatakdawala, H. and S. A. Uhland. 2011. Hydrogen peroxide mediated transvaginal drug delivery. International journal of pharmaceutics 409:121-127.

Fricke, P., D. Caraviello, K. Weigel, and M. Welle. 2003. Fertility of dairy cows after resynchronization of ovulation at three intervals following first timed insemination. Journal of dairy science 86:3941-3950.

Giordano, J., P. Fricke, J. Guenther, G. Lopes, M. Herlihy, A. Nascimento, and M. Wiltbank. 2012a. Effect of progesterone on magnitude of the luteinizing hormone surge induced by two different doses of gonadotropin-releasing hormone in lactating dairy cows. Journal of dairy science 95:3781-3793.

Giordano, J., M. Wiltbank, J. Guenther, R. Pawlisch, S. Bas, A. Cunha, and P. Fricke. 2012b. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. Journal of dairy science 95:639-653.

Han, K., J. S. Park, Y. B. Chung, M. J. Lee, D. C. Moon, and J. R. Robinson. 1995. Identification of enzymatic degradation products of luteinizing hormone releasing hormone (LHRH)/[D-Ala 6] LHRH in rabbit mucosal homogenates. Pharmaceutical research 12:1539-1544.

Hussain, A. and F. Ahsan. 2005. The vagina as a route for systemic drug delivery. Journal of controlled release 103:301-313.

Ivanov, A. I., A. Nusrat, and C. A. Parkos. 2005. Endocytosis of the apical junctional complex: mechanisms and possible roles in regulation of epithelial barriers. Bioessays 27:356-365.

Künnemeyer, R., P. Cross, C. Bunt, D. Carnegie, and M. Rathbone. 2004. Electronically controlled, intravaginal drug delivery. Proceedings of the Institution of Mechanical Engineers, Part B: Journal of Engineering Manufacture 218:1409-1415.

Lamb, G., C. Dahlen, J. Larson, G. Marquezini, and J. Stevenson. 2010. Control of the estrous cycle to improve fertility for fixed-time artificial insemination in beef cattle: a review. Journal of animal science 88:E181-E192.

Lima, F., E. Ribeiro, R. Bisinotto, L. Greco, N. Martinez, M. Amstalden, W. Thatcher, and J. Santos. 2013. Hormonal manipulations in the 5-day timed artificial insemination protocol to optimize estrous cycle synchrony and fertility in dairy heifers. Journal of dairy science 96:7054-7065.

Littell, R. C., W. W. Stroup, G. A. Milliken, R. D. Wolfinger, and O. Schabenberger. 2006. SAS for mixed models. SAS institute.

Lucy, M. C. and J. S. Stevenson. 1986. Gonadotropin-releasing hormone at estrus: luteinizing hormone, estradiol, and progesterone during the periestrual and postinsemination periods in dairy cattle. Biology of Reproduction 35:300-311.

Macmillan, K. and A. Peterson. 1993. A new intravaginal progesterone releasing device for cattle (CIDR-B) for oestrous synchronisation, increasing pregnancy rates and the treatment of post-partum anoestrus. Animal Reproduction Science 33:1-25.

Macmillan, K., V. Taufa, D. Barnes, and A. Day. 1991. Plasma progesterone concentrations in heifers and cows treated with a new intravaginal device. Animal Reproduction Science 26:25-40.

Millar, R. P. 2005. GnRHs and GnRH receptors. Animal reproduction science 88:5-28.

Moenter, S. M., A. Caraty, and F. J. Karsch. 1990. The estradiol-induced surge of gonadotropin-releasing hormone in the ewe. Endocrinology 127:1375-1384.

Moreira, F., C. Orlandi, C. Risco, R. Mattos, F. Lopes, and W. Thatcher. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. Journal of Dairy Science 84:1646-1659.

Okada, H., I. Yamazaki, Y. Ogawa, S. Hirai, T. Yashiki, and H. Mima. 1982. Vaginal absorption of a potent luteinizing hormone-releasing hormone analog (leuprolide) in rats I: Absorption by various routes and absorption enhancement. Journal of pharmaceutical sciences 71:1367-1371.

Okada, H., I. Yamazaki, T. Yashiki, and H. Mima. 1983. Vaginal absorption of a potent luteinizing hormone-releasing hormone analogue (leuprolide) in rats II: Mechanism of absorption enhancement with organic acids. Journal of Pharmaceutical Sciences 72:75-78.

Picard-Hagen, N., G. Lhermie, S. Florentin, D. Merle, P. Frein, and V. Gayrard. 2015. Effect of Gonadorelin, lecirelin, and Buserelin on LH surge, ovulation, and progesterone in cattle. Theriogenology 84:177-183.

Price, C., B. Morris, and R. Webb. 1987. Reproductive and endocrine effects of active immunization against a testosterone conjugate in the heifer. Journal of reproduction and fertility 81:149-160.

Pulley, S. L., D. Keisler, and J. Stevenson. 2015. Concentrations of luteinizing hormone and ovulatory responses in dairy cows before timed artificial insemination. Journal of dairy science 98:6188-6201.

Pursley, J., M. Mee, and M. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF 2α and GnRH. Theriogenology 44:915-923.

Pursley, J., M. Wiltbank, J. Stevenson, J. Ottobre, H. Garverick, and L. Anderson. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus. Journal of Dairy Science 80:295-300.

Rantala, M., O. Peltoniemi, T. Katila, and J. Taponen. 2009. Effect of GnRH dose on occurrence of short oestrous cycles and LH response in cyclic dairy heifers. Reproduction in domestic animals 44:647-652.

Rathbone, M. J., K. L. Macmillan, K. Inskeep, S. Burggraaf, and C. R. Bunt. 1998. Fertility regulation in cattle. Journal of controlled release 54:117-148.

Richardson, J. L. and L. Illum. 1992. (D) Routes of delivery: Case studies:(8) The vaginal route of peptide and protein drug delivery. Advanced drug delivery reviews 8:341-366.

Souza, A., H. Ayres, R. Ferreira, and M. Wiltbank. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology 70:208-215.

Stevenson, J. and S. Pulley. 2016. Feedback effects of estradiol and progesterone on ovulation and fertility of dairy cows after gonadotropin-releasing hormone-induced release of luteinizing hormone. Journal of dairy science 99:3003-3015.

Stewart, K., W. Flowers, G. Rampacek, D. Greger, M. Swanson, and H. Hafs. 2010. Endocrine, ovulatory and reproductive characteristics of sows treated with an intravaginal GnRH agonist. Animal reproduction science 120:112-119.

Viudes-de-Castro, M., R. Lavara, F. Marco-Jiménez, C. Cortell, and J. Vicente. 2007. Ovulation induced by mucosa vaginal absorption of Buserelin and triptorelin in rabbit. Theriogenology 68:1031-1036.

Wiltbank, M. C. and J. R. Pursley. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. Theriogenology 81:170-185.

SECTION III

OVERALL CONCLUSIONS AND FUTURE RESEARCH

1. Strategies to maximize reproductive performance of non-pregnant previously inseminated lactating dairy cows based on ovarian status at nonpregnancy diagnosis

1.1 Overall conclusions

The objective of the first experiment described in Chapter II of Section I of this dissertation was to have a better understanding of ovarian dynamics in previously inseminated dairy cows. More specifically, a major interest was to elucidate the impact of embryo mortality on ovarian function because of the relevance of ovarian responses to the success of resynchronization of ovulation protocols for second and greater AI services. Embryo mortality was diagnosed using mRNA expression of interferon stimulated genes (ISG) on peripheral white blood cells and circulating concentration of pregnancy specific protein B (PSPB). Cows with circulating PSPB concentration similar to those of pregnant cows but with no embryo visualized through transrectal ultrasonography (TUS) from Day 14 to 42 after artificial insemination (AI) were considered to have undergone late embryo mortality (LEM; after Day 24 after AI). Conversely, cows that did not meet this criterion but had ISG mRNA expression fold changes indicative of the presence of an embryo from 18 to 22 days after AI were considered to have undergone early embryo mortality (EEM; before Day 24 after AI). This was the first time this methodology was used to diagnose embryo mortality in cattle. Most published research regarding the impact of embryo mortality on the estrous cycle was performed based on the assumption that cows undergoing EM had extended luteal phases. This diagnostic method is not

accurate, because the presence of a corpus luteum (CL) or elevated concentration of progesterone (P4) are not indicative of the presence of an embryo. Although we did not have a gold standard method to detect the presence of an embryo, using ISG mRNA expression as diagnostic method allowed us to indirectly detect embryos through an embryo-derived product like Interferon-τ. Thus, unlike previous research in which the outcome of embryo mortality (i.e., extended luteal phases) rather than evidence of the presence or absence of an embryo was used as diagnostic method, the methods used in our experiment provided a better indication of the actual incidence of embryo mortality and its consequences for ovarian dynamics. In contrast to what has been described in the literature, we observed delayed luteal regression in cows that underwent LEM but not in cows undergoing EEM. Therefore, we concluded that the embryo needs to survive beyond Day 22 after conception to cause significant alterations to the estrous cycle, in particular prevent luteolysis. Certainly, this hypothesis should be tested using a larger sample size as we had a limited number of cows in our experiment. Further, we did not observe significant alterations to follicular growth and regression patterns, or circulating concentration of estradiol (E2) around the onset of luteolysis and ovulation. Therefore, we concluded that embryo mortality had no major effect on ovarian function other than delaying luteal regression in cows that underwent LEM.

In the same experiment, we also investigated follicular and luteal dynamics at the approximate time points when resynchronization of ovulation programs are commonly initiated in commercial dairy farms (i.e., 24, 32, and 38 days after AI; Appendix 1). Considering all inseminated cows that did not have an embryo present 32 days after AI (i.e., nonpregnant and embryo mortality cows) we observed that most cows had an active follicle (92%; ≥10 mm in diameter and in the growing or static phase of development) and an active CL (84%; P4 ≥1

ng/mL) 24, 32, and 38 days after AI. Only 30 and 57% of the cows, however, had a mature CL (>6 days old) 24 and 32 days after AI, respectively. Thus, we concluded that at those time points, most previously inseminated cows had similar ovarian structures, and to some extent at a similar stage of development than cows at the time of prostaglandin F2α (PGF2α) treatment of Ovsynch-like protocols (i.e., presence of a putative ovulatory follicle and an active CL). Therefore, we hypothesized that these cows could be treated with PGF2α to induce luteolysis and then Gonadotropin Releasing Hormone (GnRH) to induce ovulation before TAI without receiving GnRH to induce a new follicular wave before induction of luteolysis. Our reasoning was that most cows 32 ± 3 d after AI would have a dominant follicle generated through spontaneous follicular wave turnover, capable of continuing to grow as well as a CL responsive to PGF2α. Removing the GnRH treatment to induce a new follicular wave before induction of luteolysis would eliminate one of the main inconveniences of treating cows with GnRH 25 ± 3 d after AI (around the time of expected estrus expression after a previous insemination) that is suppress estrus expression. Therefore, dairy farms that are successful detecting cows in estrus and observe reasonable P/AI to those AI services can benefit from both insemination of cows at detected estrus and a short interbreeding interval for most nonpregnant cows that need TAI. Another important benefit of the strategy without the initial GnRH treatment would be not treating pregnant cows with GnRH before their pregnancy status is determined. This is important for reducing not only treatment cost but also the burden of finding and treating cows which is both labor intensive and disrupts normal cow behavior.

Based on the observations from this research, we designed a new management strategy for second and greater AI services in which cows were differentially treated based on the ovarian structures present at the time of NPD. Cows with an ovarian status similar than that observed at

the time of the PGF2α injection of Ovsynch-like protocols (i.e., had a functional CL responsive to PGF2α and an ovarian follicle capable of continuing to growth and ovulate in response to GnRH) received a short resynchronization of ovulation protocol consisting of: induction of luteolysis with PGF2α, 24 h later PGF2α, 32 h later GnRH, and 16 to 18 h later TAI. We named this protocol Short Resynch (SR) because cows were inseminated within 3 d of the beginning of the protocol. A reproductive management program that included SR as the main strategy to resynchronize cows was tested in the experiments described in Chapters III and VI from Section I of this dissertation. In the first experiment, our objectives were to test the impact of removing the GnRH treatment to induce a new follicular wave before induction of luteolysis in the SR protocol on estrus expression before NPD and pregnancy per AI (P/AI). We hypothesized that removing the GnRH 25 ± 3 d after AI would result in more cows inseminated at detected estrus than when GnRH is given to induce a new follicular wave. We also hypothesized that there would be no difference in the overall proportion of pregnant cows (cows inseminated at estrus + cows inseminated at TAI) because pregnancies generated through inseminations at detected estrus would compensate for the potential reduction in P/AI in cows that did not receive GnRH 25 ± 3 d after AI. Therefore, we compared in a randomized controlled experiment the SR treatment to the same protocol but including GnRH treatment to induce a new follicular wave. In both treatments NPD was performed 32 ± 3 days after AI and cows bearing a CL and a putative ovulatory follicle received two PGF2α treatments 24 hours apart, GnRH 32 hours after the second PGF2α, and TAI 16 hours later. Cows with no CL and/or a putative ovulatory follicle (i.e. ≥10 mm) at NPD were not expected to respond to this treatment and therefore received the Ovsynch protocol with two PGF2α treatments and P4 supplementation. We decided to use two PGF2α treatments because in the experiment presented in Chapter II we observed that a large

proportion of cows did not have a mature CL at the time of NPD. We expected these cows to have a poor response to a single PGF2α treatment. As expected (Chapter III), we observed more cows inseminated at detected estrus before NPD in the group that did not receive GnRH treatment seven days before NPD. Conversely, conception risk for cows that had a CL and a putative ovulatory follicle at NPD was greater for cows that received GnRH 25 ± 3 d after AI. Our explanation for the reduced P/AI in cows in SR was that the putative ovulatory follicle at the time of NPD was on average 1.9 days older than in cows that received GnRH, most likely because of poor synchrony of follicular wave turnover. In these cows the extended period of dominance of the ovulatory follicle may have negatively affected oocyte and embryo quality. On the other hand, there was no difference in P/AI among cows inseminated at detected estrus, cows with no CL at NPD, or when considering all AI services (estrus AI + TAI) together. We concluded that the SR program was a valid alternative to reduce the interbreeding interval for TAI services and not reduce the proportion of cows inseminated upon estrus detection after a previous insemination. A limitation of this experiment was the re-randomization of cows to the experimental treatments after each AI service precluding evaluation of time to pregnancy during lactation.

Therefore, the experiment described in Chapter IV of Section I of this dissertation was designed to test the hypothesis that the SR program would reduce time to pregnancy during lactation as compared to use of a program based on blanket enrollment in the Ovsynch protocol for resynchronization of ovulation 32 ± 3 d after AI (i.e., Day 32-Resynch). Another objective was to provide additional evidence that reasonable conception risk could be obtained with the SR program. We expected the SR program to reduce time to pregnancy due to: (1) no reduction in estrus expression after the previous AI service, (2) reduced interbreeding interval for cows with a

CL at nonpregnancy diagnosis, and (3) improved P/AI of cows with no CL at nonpregnancy diagnosis when compared with blanket use of the Day 32-Resynch protocol. We decided to use this protocol for the control group because it is the most widely used protocol for resynchronization of ovulation in the U.S. Cows were randomly assigned to treatments after the first service postpartum. Afterwards, cows were followed for 210 days or until cows left the herd. Although overall P/AI was not different among treatments, the fact that cows with a CL and a putative ovulatory follicle at NPD (~70%) had a seven day reduction in the interbreeding interval along with greater P/AI for cows with no CL at NPD, resulted in greater hazard of pregnancy. Although an economic analysis was not performed in this experiment, we speculate that the reduction in time to pregnancy (i.e., 16 d reduction in median days) and reduced proportion of nonpregnant cows at the end of lactation would have a greater positive impact on cow profitability than the potential additional cost of the SR program. As expected, P/AI was greater for the SR group among cows with no CL and/or a putative ovulatory follicle at NPD. In contrast to our observations from the previous experiment, P/AI was not different among cows with a CL at NPD. We speculate that the negative impact of not synchronizing the follicular wave with the first GnRH treatment did not affect P/AI because of two reasons. First, the initial GnRH treatment of the Day 32-Resynch protocol was given 32 and not 25 days after AI hence, a large proportion of cows expected to have high fertility were inseminated at estrus before NPD rather than through TAI as it may have happened if they received GnRH on Day 25. Second, the use of an additional PGF2α treatment to increase luteal regression before TAI in the SR treatment.

In summary, the experiments presented in Section I of this dissertation showed that EEM does not cause major alterations to the ovarian dynamics of lactating dairy cows, whereas LEM

leads to extended luteal phases but not major alterations to the follicular wave dynamics. In addition, the great proportion of previously inseminated cows that present an active CL and a putative ovulatory follicle 32 ± 3 days after AI provides an opportunity to use resynchronization of ovulation programs such as the SR program which did not interfere with inseminations at detected estrus and reduced time to pregnancy after the first service.

1.2 Future research

In the experiment performed in Chapter I of Section I we tested a new method to detect the presence of an embryo around the period of maternal recognition of pregnancy and thereafter classified cows as undergoing EEM (18 to 23 days after AI) or LEM (≥ 24 days after AI). We observed that the occurrence of EEM did not have a major impact on ovarian dynamics, whereas LEM resulted in delayed luteolysis. After this experiment, many questions regarding ovarian dynamics in cows suffering embryo mortality remain unanswered. For example: (1) is luteolysis triggered by the same mechanism(s) in cows that undergo maternal recognition of pregnancy but later lose their pregnancy than in cows that do not undergo maternal recognition of pregnancy?, (2) up to how many days after fertilization is IFNT produced and for how many days is it required to, or capable of preventing luteolysis?, (3) is there a molecular signal or a set of molecular signals that prevent luteolysis beyond the period of maternal recognition of pregnancy in cattle?, and (4) is uterine receptivity to an embryo compromised after early and/or late embryo mortality in cattle?

To determine whether (first question) luteolysis is triggered through the same mechanisms after maternal recognition of pregnancy or during the estrous cycle in cows with no exposure to an embryo around the period of maternal recognition of pregnancy, an experiment

could be performed to monitor ovarian dynamics, expression of hormone receptors in the endometrium, PGF2α secretion, and corpus luteum function in cows exposed or not exposed to a conceptus and/or IFNT. The experiment would include three groups: (1) non-inseminated cyclic cows, (2) pregnant cows with induced embryo loss after Day 25 of pregnancy, and (3) non-inseminated cows with maternal recognition of pregnancy simulated by intrauterine infusion of recombinant IFNT from Day 14 to 25 after AI. In the cyclic group, blood samples, uterine biopsies, and CL biopsies would be collected at 48 h intervals from Day 12 to Day 20 after induction of ovulation. In the other two treatments the same samples would be collected every 48 hours from Day 25 to Day 33 after induction of ovulation. At each sampling point transrectal ultrasonography (B-mode and color Doppler) would be performed to monitor size and blood flow of ovarian structures and uterine content. Circulating concentrations of estradiol and progesterone would be measured in blood samples to monitor follicular and luteal function. Analysis of both protein and mRNA abundance for E2, oxytocin, and P4 receptors would be performed in endometrial tissue samples. In CL biopsy samples, both protein and mRNA abundance analysis would be performed for oxytocin and markers of steroidogenic function (steroidogenic acute regulatory protein and 3β-hydroxysteroid dehydrogenase), apoptosis (BAX and BCL2), inflammation (tumor necrosis factor-α), and vascular remodeling (vascular endothelial growth factor). Moreover, on the days of the first, third, and fifth biopsy, blood samples would be collected every 30 minutes during four hours prior to biopsy collection to measure circulating concentrations of PGFM (a PGF2α metabolite). As described in Chapter I of Section I, the onset and inhibition of luteolysis are regulated through interactions among the ovary, CL, endometrium, and embryo. The regulation of this process is governed by the abundance of E2, oxytocin, and P4 receptors in the endometrium, which are regulated by E2, P4,

oxytocin, and IFNT. Pulsatile secretion of PGF2α is required for luteolysis to occur. This pulsatile release is triggered by oxytocin, therefore there must be a down regulation of the P4 receptor and an upregulation of the oxytocin receptor in the endometrium for luteolysis to occur. During maternal recognition of pregnancy, the oxytocin receptor is downregulated in the endometrium due to the action of IFNT. Whether the mechanism that leads to luteolysis after embryo mortality during or after the period of maternal recognition of pregnancy follows the same pathways and timing as in a normal estrous cycle is unknown. I hypothesize that the presence of an embryo along with embryonic membranes and secretions will alter the way in which the luteolytic mechanism is established as compared with a normal estrous cycle. This experiment would allow to elucidate the physiological and molecular mechanisms that lead to delayed luteolysis in cows that suffer late embryonic mortality.

A simple experiment can also be conducted to determine the relationship between duration of IFNT exposure and luteal lifespan. Cows would be randomly assigned to receive intrauterine infusions of recombinant IFNT or saline solution from Day 14 to 35 after induction of ovulation or the time of CL regression, whatever occurs first. Daily blood sampling would be performed to determine P4 and E2 concentrations. At the same time peripheral white blood cells would be harvested to analyze mRNA expression of ISG to monitor the systemic response to IFNT. Moreover, uterine biopsies would be obtained from a subgroup of cows to measure protein and mRNA expression of E2, P4, and oxytocin receptors in the endometrium because these are the key regulators of luteolysis. The main objectives would be to determine if there is a maximum number of days after ovulation that IFNT can block the luteolytic mechanism. Further, to explore the potential influence of other molecules on maternal recognition of pregnancy, a third group of cows may be included to receive intrauterine infusions of embryo lysates in which

IFNT has been removed or blocked using antibodies. Inclusion of a treatment with lysates from embryos at different stages of development may also help elucidate the effect of time on production and/or secretion of other molecules that may be involved in the prevention of luteolysis in pregnant cows.

Determining the impact of embryo mortality on subsequent fertility would be important because of the substantial proportion of cows that suffer embryo mortality and are re-inseminated immediately after NPD. Many of these cows do not undergo luteolysis before the time of NPD, and therefore it is fair to expect that the endometrium had been exposed to embryonic tissues for a prolonged period of time and a different endocrine environment than a cow which already had CL regression. If these cows receive a resynchronization of ovulation treatment that results in immediate re-insemination (e.g., Short-Resynch protocol described in Chapters III and IV) it is reasonable to speculate that the uterine environment might be different from a cow that did not have delayed luteal regression because of lack of fertilization or early embryo mortality. An experimental design to address the question of uterine receptivity after embryo loss would include two treatments. One of the treatments would consist of non-inseminated cyclic cows whereas the other group would consist of cows with induced embryo mortality. In the embryo mortality treatment, pregnancy would be diagnosed using circulating concentration of pregnancy associated glycoproteins (PAG) and transrectal ultrasonography. Thereafter, embryo mortality would be induced on Day 27 after AI. On Day 32 after synchronized ovulation cows from both groups with a CL and an active follicle would receive PGF2α, a second PGF2α treatment 24 h later, GnRH 32 hours after and timed AI 16 hours later. Any cow observed in estrus after induction of embryo mortality would be inseminated immediately. After re-insemination, pregnancy diagnosis would be performed starting at 24 days

after AI using circulating PAG concentration and by transrectal ultrasonography 32 days after AI. This pregnancy testing scheme will allow detecting pregnancy loss at an early stage of gestation. This experiment would help to elucidate, at least in part, if the presence and later death of an embryo causes alterations in uterine receptivity and further embryo survival. The main limitation of this experiment would be the large number of cows needed per treatment because the main outcome would be P/AI.

Regarding the second part of Section I, composed by Chapters III and IV, further research would be directed toward validation of the SR program. The first question that arises is how the SR program would compare to the Day 25-Resynch program (first GnRH given 25 ± 3 days after AI) for time to pregnancy and differences on the proportion of cows that receive AI at detected estrus. Although the experiment described in Chapter III showed a greater proportion of cows detected in estrus before NPD and no difference was observed in overall P/AI, experimental design limitations prevented us from testing the hypothesis that cows in the SR program would have greater insemination risk thereby, result in greater hazard of pregnancy. Therefore, an experiment similar to the one presented in Chapter IV is being conducted to test this hypothesis. Briefly, after the first service postpartum cows are randomly assigned to receive the SR program or the Day 25-Resynch protocol. For both treatments, cows with no CL ≥15 mm and follicle ≥10 mm at the time of NPD receive the Ovsynch protocol with two PGF2α treatments and P4 supplementation. Our objectives are: (1) to evaluate the effect of removing the GnRH treatment 25 ± 3 d after AI on the hazard of pregnancy after the first AI postpartum, and (2) validate results from the experiment presented in Chapter IV. Of particular interest are the effect of GnRH or no GnRH treatment on the re-insemination dynamics before NPD, and P/AI for cows with a CL ≥15 mm and follicle ≥10 mm at NPD.

In the experiments presented in Chapters III and IV we showed that the SR program generated a similar proportion of pregnant cows after NPD when compared with the Day 25 (i.e., for cows with CL at NPD) and Day 32-Resynch protocols, but greater hazard of pregnancy during lactation than the latter. However, an economic analysis is needed to determine the profitability of cows managed with these programs. Therefore, in future experiments data for milk yield, feed cost, reproductive cost, and replacements cost would be needed to conduct such analysis and determine the true impact of the reducing time to pregnancy and inseminating more cows at detected estrus on dairy herd profitability.

More questions were also generated during analysis of data from the experiment presented in Chapter III. For example, what is the fate of the dominant follicle in cows that receive GnRH seven days prior to NPD and do not ovulate? The notion that follicular turnover only happens in cows that ovulate in response to GnRH treatment in resynchronization of ovulation protocols does not necessarily match the pattern of estrus expression observed in the experiment presented in Chapter III. Even though only 50% of the cows ovulated in response to this GnRH treatment, there was a clear difference in the pattern of estrus expression after Day 25 compared to cows that did not receive GnRH treatment. Therefore, one of my hypothesis is that in cows that do not ovulate, the LH surge elicited by the GnRH treatment suppresses E2 production in the dominant follicle. As a consequence, this would suppress estrus expression and allow a rise in circulating concentration of follicle stimulating hormone (FSH) which may lead to the emergence of a new follicular wave. An observational study to monitor ovarian function, and FSH and LH concentrations after GnRH treatment in cows that do not ovulate would be performed. Cows in diestrus (seven days after ovulation) would be used rather than cows 25 days after a previous ovulation because of the great variability expected for stage of follicle and CL

development as well as P4 and E2 concentrations. Thus, cows would be synchronized with the Ovsynch protocol and receive GnRH seven days later. Only cows that ovulate to this GnRH would be eligible to receive another GnRH treatment seven days later. Transrectal ultrasonography and ovarian mapping of all structures ≥3 mm would be performed at 12 hour intervals starting one day before the last GnRH treatment until seven days after GnRH. Blood samples to measure E2, P4, LH, and FSH concentrations would be collected every two hours from -2 to 12 hours after treatment, every 6 hours until 24 hours after treatment, and then every 12 hours until 7 days after treatment. My hypothesis is that in cows that fail to ovulate after the GnRH treatment, the GnRH-induced LH surge will cause the dominant follicle to stop producing E2, hence allowing an increase in circulating concentration of FSH which will lead to the emergence of a new follicular wave. This experiment would allow description of follicular wave dynamics and hormonal profiles in cows that have a dominant follicle but fail to ovulate after GnRH treatment. In addition, because there would be cows that do ovulate, we would be able to compare differences in the response variables between cows that do and do not ovulate.

2. The vagina as a route of hormone delivery for synchronization of ovulation in cattle

2.1 Overall conclusions

The objectives of the experiments presented in Section II of this dissertation were to test the feasibility of inducing an LH surge and luteal regression through intravaginal (IVG) delivery of GnRH and PGF2α, respectively. The long-term objective of this line of research is to develop electronically controlled automated IVG drug delivery devices. Such devices could be programed to release the required type and dose of hormones at predefined time points to synchronize ovulation and be removed immediately before TAI. These devices would allow to:

(1) maximize compliance in timing and dosage of drug administration, (2) minimize labor requirements for treatments, (3) minimize cow time budget disruption and number of injections, (4) better mimic physiological patterns of hormone release, and (5) tailor treatments to individual cows.

In Chapter II of Section II, two experiments were performed to test the feasibility of inducing luteal regression through IVG administration of PGF2α to lactating dairy cows. Progesterone concentration dynamics after IVG administration of different doses of PGF2α (25, 50, and 100 mg of Dinoprost) were compared to a negative control (IVG saline solution) and a positive control group [25 mg Dinoprost administered intramuscular (i.m.)]. All IVG treatments induced an initial decline in circulating P4 concentration. Nevertheless, we observed that a substantial proportion of cows had an initial decline in P4 concentration followed by return to pretreatment levels. Therefore, our conclusion from this first experiment was that although luteolysis was triggered in the treatments receiving PGF2α through IVG delivery, the stimulus was insufficient to cause complete luteal regression. Thus, we performed a second experiment in which we added a treatment that received two IVG treatments (25 mg Dinoprost) 12 hours apart. As observed in the first experiment, cows that received a single IVG treatment of PGF2α had an initial decline in P4 concentration followed by a rebound. Conversely, cows that received two IVG treatments 12 hours apart had a P4 concentration pattern very similar to cows in the positive control group. In these cows, P4 concentration dropped below 0.5 ng/mL by 36 hours after treatment and remained at these levels until 96 h after treatment. Results from these experiments supported the hypothesis that it is feasible to induce luteal regression in lactating dairy cows through IVG administration of PGF2α, and that two PGF2α treatments 12 hours apart are required to prevent a rebound in P4 concentration after the initial treatment.

The experiments described in Chapter III of Section II, had the objective of testing the feasibility of inducing an LH surge through IVG administration of GnRH. We aimed to trigger a surge of LH of similar magnitude and timing than observed after i.m. treatment with GnRH. In the first experiment circulating concentration of LH was measured after IVG administration of three different doses (100, 500, and 1,000 µg of Gonadorelin) of GnRH. A negative control (IVG saline) and a positive control (100 µg Gonadorelin i.m.) group were also included. None of the IVG treatments elicited a surge of LH, and only the highest dose caused a slight increase in circulating LH concentration. We concluded that GnRH was either not absorbed or degraded before absorption, and therefore did not reach the blood stream and the pituitary gland. The fact that there was a discrete response to the 1,000 µg dose suggested that at least a portion of the dose delivered was absorbed. Therefore, we performed a second experiment to test the hypothesis that adding an absorption enhancer would increase vaginal absorption of GnRH. In this experiment, we also included a more potent GnRH analogue (Buserelin) to test the hypothesis that a more potent analogue may be more effective to induce a surge of LH because inefficient absorption could be offset by greater potency (i.e., less amount needed to trigger the desired response). Therefore, using the same positive and negative controls as in the previous experiment, we measured circulating LH concentration after IVG administration of 1,000 µg of Gonadorelin and 80 µg of Buserelin with and without the inclusion of citric acid as an absorption enhancer. As in the first experiment, IVG administration of GnRH alone failed to elicit an LH surge. On the other hand, treatments including citric acid elicited an LH surge of similar magnitude as the i.m. treatment. Although there was no difference in area under the curve or peak LH concentration between the positive control and treatments including citric acid, there was a delay of 40 (Gonadorelin) to 80 (Buserelin) minutes in time to peak LH concentration.

This, and the fact that GnRH doses 10 fold greater did not cause an LH surge of greater magnitude after IVG than i.m. delivery suggest that either IVG absorption was less efficient and/or that GnRH was degraded by enzymes present in the vagina. Because we did not test the lower doses with the inclusion of citric acid, we can only speculate that these would not have had a similar response. We concluded that it is feasible to induce an LH surge through IVG administration of GnRH when citric acid is included as an absorption enhancer. We also concluded that using more potent analogues does not necessarily overcome poor absorption.

The experiments presented in Chapters II and III of Section II supported the hypotheses that both luteal regression and an LH surge can be triggered through IVG administration of PGF2α and GnRH, respectively. Despite this, the experimental design of these experiments was inadequate to test potential differences between the IVG and i.m. treatments on: (1) risk of complete luteal regression, (2) risk of ovulation, and (3) time to ovulation after treatment. Thus, further research is needed to determine the best time, dose, and number of treatments needed to achieve the same or better results than those observed when cows receive i.m. GnRH and PGF2α.

2.2 Future research

Further research related to this line of investigation should be directed towards: (1) evaluating efficacy of IVG administration of PGF2α to induce complete luteal regression with a larger number of cows, (2) evaluate efficacy of IVG administration of GnRH to induce ovulation with a larger number of cows and potential differences on time to ovulation compared to i.m. administration, (3) identify the best PGF2α and GnRH analogues, doses, and vehicles to effectively induce luteal regression and ovulation after IVG delivery, and (4) develop and test electronically controlled automated IVG drug delivery devices.

We are currently performing an experiment to compare the risk of undergoing complete luteolysis after a single i.m. treatment of PGF2α or two IVG treatments 12 hours apart. Briefly, non-inseminated lactating dairy cows are randomly assigned to receive the i.m. or IVG PGF2α (500 µg of Cloprostenol) treatments seven days after a GnRH treatment. Transrectal ultrasonography of ovarian structures and blood sampling to measure P4 concentration are performed at the time of: GnRH treatment, PGF2α treatment, 24, 48 and 72 hours (at this time cows receive a second GnRH treatment) after treatment. Our main objective is to test the hypothesis that there is no difference in the proportion of cows undergoing complete luteal regression after i.m. or IVG administration of PGF2α. Further, we will also monitor physical activity patterns to compare estrous behavior between cows that receive PGF2α through the IVG and i.m. route of administration.

Another experiment should be performed to test the efficacy of IVG administration of GnRH to induce ovulation and describe time to ovulation after treatment. A possible experimental design would include five treatments: a positive control (100 µg Gonadorelin i.m.), a negative control (saline IVG), and an IVG treatment with 100, 500 and 1,000 µg Gonadorelin and 10% citric. Lactating dairy cows would be synchronized with the Ovsynch protocol and treated with PGF2α seven days later, at this time, cows would be randomly assigned to receive treatments. Transrectal ultrasonography and ovarian mapping would be performed 24 hours before treatment, and every 4 hours from the time of treatment until 48 hours after to determine the time of ovulation, which would be confirmed seven days after treatment through transrectal ultrasonography and circulating P4 concentration. This experiment would allow to identify any difference in risk and timing of ovulation after IVG instillation of different doses of GnRH compared to i.m. administration. The large number of cows needed to compare ovulation risk

would not allow to perform the frequent blood sampling needed to detect the presence and magnitude of an LH surge.

The development of electronically controlled automatic drug delivery device is beyond my area of expertise and was not attempted during my PhD program.

APPENDIX

Appendix 1. Proportion of cows bearing an active corpus luteum, a mature corpus luteum, an active follicle, and largest active follicle diameter 24, 32, and 38 days after AI for cows that received Sham insemination and cows nonpregnant cows inseminated with semen.

Days after AI	Item	Cyclic[1]	Nonpregnant[1]	*P* value
24	ACL[2] (%; n/n)	33 (5/15)	74 (14/37)	0.76
	MCL[3] (%; n/n)	0 (0/15)	30 (11/37)	<0.001
	AFOL[4] (%; n/n)	73 (11/15)	81(30/37)	0.54
	AFOL[5] diam. (mm)	15.7 ± 1.1	15.4 ± 0.6	0.78
32	ACL (%; n/n)	93 (14/15)	84 (31/37)	0.38
	MCL (%; n/n)	80 (12/15)	57 (21/37)	0.13
	AFOL (%; n/n)	100 (15/15)	92 (34/37)	0.97
	AFOL diam. (mm)	20.4 ± 0.9	15.7 ± 0.6	<0.001
38	ACL (%; n/n)	87 (13/15)	86 (32/37)	0.98
	MCL (%; n/n)	87 (13/15)	86 (32/37)	0.99
	AFOL (%; n/n)	93 (14/15)	100 (37/37)	0.97
	AFOL diam. (mm)	14.6 ± 0.7	15.3 ± 0.4	0.35

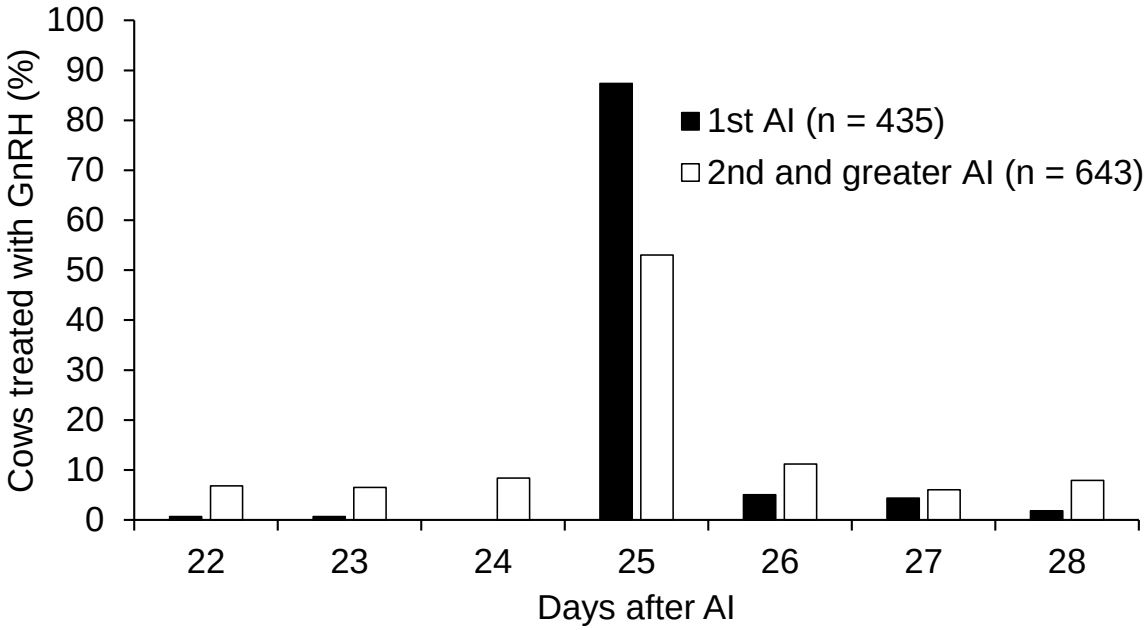
[1]Lactating Holstein cows were synchronized using the Presynch-Ovsynch protocol and at the time of AI (Day = 0) randomly assigned to receive AI with extender only (Cyclic) or regular semen in a ratio of approximately 1 to 5. Transrectal ovarian ultrasonography was conducted and blood samples were collected at the time of AI and then 2 and 7 days after AI to verify ovulation and formation of a functional corpus luteum (progesterone ≥ 1 ng/mL) based on circulating concentrations of progesterone and estradiol. From Day 14 to 42, transrectal ultrasonography was conducted every 24 h to record the presence and size (diameter) of all ovarian structures and evaluate uterine content. In addition, blood samples were collected every 48 h for determination of circulating concentrations of progesterone. Cows where no embryo was visualized through transrectal ultrasonography were considered as nonpregnant.

[2]ACL: active corpus luteum (progesterone ≥ 1).

[3]MCL: mature corpus luteum (≥ 6 days after ovulation).

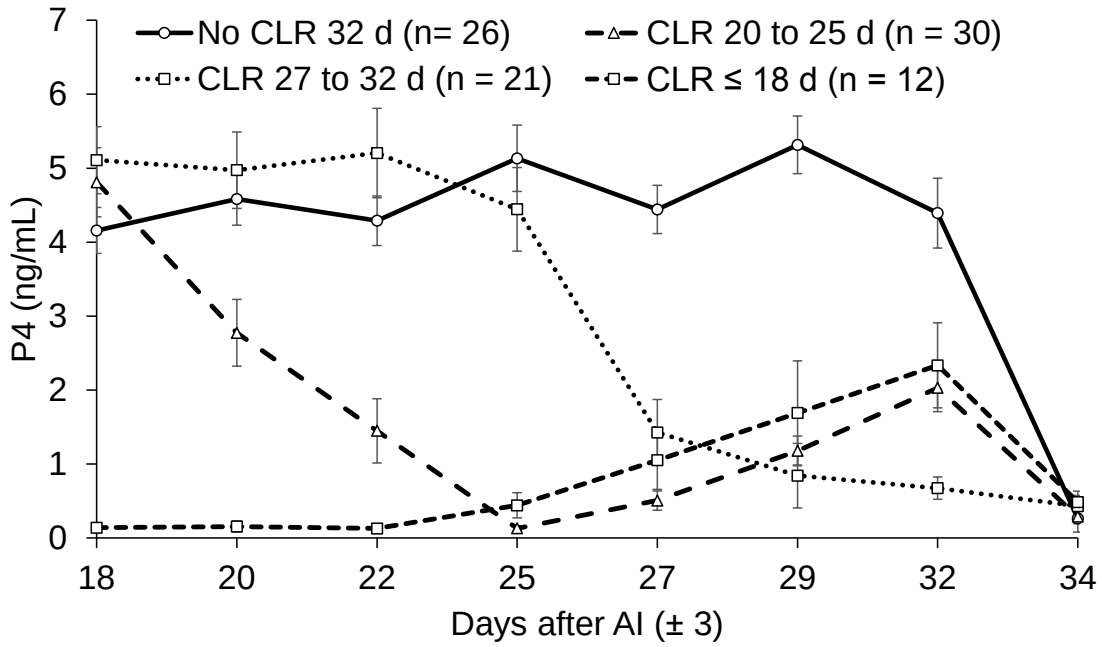
[4]AFOL: active follicle (diameter ≥ 10 mm and in growing or static phase).

Appendix 2.



Appendix 2. Distribution of cows in the G25 treatment (Section I, Chapter III) by days after first or second and greater AI services when cows received GnRH treatment 25 ± 3 d after AI.

Appendix 3.



Appendix 3. Progesterone concentration dynamics from enrollment until the time of the GnRH treatment before TAI in cows from the G25 and NoG25 treatments (Section I, Chapter III) combined. Cows were grouped according to the approximate day of complete luteal regression (**CLR**; P4 ≤0.5 ng/mL) after AI: CLR before 18 d after AI, CLR from 20 to 25 d after AI, CLR from 27 to 32 d after AI, no CLR by 32 d after AI.