

**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Exogenous hormonal manipulation to increase reproductive efficiency in
dairy cows**

Pedro Leopoldo Jerônimo Monteiro Junior

Thesis presented to obtain the degree of Doctor in
Science. Area: Animal Science and Pastures

**Piracicaba
2014**

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Veterinarian

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RESUMO

Manipulação hormonal exógena para aumentar a eficiência reprodutiva de vacas leiteiras

Nos últimos anos, em rebanhos leiteiros, foi observado um aumento acentuado da produtividade acompanhado de uma diminuição da eficiência reprodutiva das vacas lactantes. Diversos fatores, como o aumento da incidência de doenças, da suscetibilidade ao estresse térmico e da ingestão de matéria seca, têm sido atribuídos como possíveis causas para esse decréscimo na fertilidade. Aumento da ingestão de matéria seca está associado maior fluxo sanguíneo hepático, que por sua vez está relacionado com aumento da metabolização hepática de diversas moléculas, entre elas os hormônios esteroides. Tendo em vista o alto metabolismo destes hormônios nas vacas leiteiras, foram realizados seis estudos, que na presente tese estão divididos em três capítulos, envolvendo suplementação hormonal em vacas leiteiras lactantes. O primeiro capítulo teve como objetivo aumentar a taxa de sincronização de vacas leiteiras ao protocolo de inseminação artificial em tempo fixo (IATF) à base de estradiol (E2)/progesterona (P4). Para isso foram realizados dois experimentos, sendo o primeiro (n = 44 vacas) utilizando dose de 2.0 ou 3.0 mg de benzoato de estradiol (BE) associado a um dispositivo de P4, no início do protocolo. No segundo (n = 82 vacas), foi realizada uma pré-sincronização com GnRH para iniciar o protocolo de IATF em diferentes estágios foliculares: recrutamento vs. dominância. Avaliações ultrassonográficas e dosagens hormonais foram feitas. Outros 4 experimentos, descritos no segundo (n = 1070 vacas) e no terceiro (n = 1498 vacas) capítulo, foram desenvolvidos para avaliar o efeito da suplementação de P4 após a ovulação em vacas leiteiras. Nesses estudos, foram avaliados os efeitos desta suplementação na formação e função do corpo lúteo (CL), na expressão de genes estimulados por interferon (ISG), na fertilidade de vacas submetidas a IA, através da observação de estro ou de protocolo de IATF, e em receptoras de embrião. A dose de 3.0 mg de BE, além de não aumentar a taxa de sincronização da emergência de uma nova onda folicular, induziu luteólise em um maior número de vacas que a dose de 2.0 mg. Independente da fase do ciclo estral, no início do protocolo a base de E2/P4, houve falhas na indução na sincronização da emergência e de ovulação. A suplementação com P4 após a ovulação não alterou a formação e função do CL, mas também não aumentou a expressão de ISG. Vacas submetidas a IA após detecção de estro ou submetidas à IATF em protocolos a base de E2/P4 não apresentaram aumento na fertilidade, no entanto quando submetidas ao protocolo de IATF à base de GnRH foi observado em torno de 8% de incremento de fertilidade. Contudo, receptoras de embrião suplementadas com P4 tiveram menor fertilidade. Assim, concluiu-se que independente da dose de EB ou do momento do ciclo estral em que se inicia o protocolo de IATF à base de E2/P4 há falhas de emergência de uma nova onda e/ou de ovulação ao final do protocolo. Além disso, dependendo do protocolo utilizado, a suplementação com P4 pós-ovulação pode aumentar a fertilidade de vacas submetidas à IATF, contudo compromete a fertilidade de receptoras de embrião.

Palavras-chave: Estradiol; Inseminação artificial; Progesterona; Sincronização; Transferência de embrião

ABSTRACT

Exogenous hormonal manipulation to increase reproductive efficiency in dairy cows

In recent years, in dairy cattle, while it was observed a gradual increase in productivity, a decrease occurred in the reproductive efficiency. Several factors, such as increased incidence of diseases, higher susceptibility to heat stress and increase of dry matter intake, have been awarded as possible causes for the decrease in fertility. Increased dry matter intake is associated with increased liver blood flow, which is associated with an increase in liver metabolism of steroid hormones. Given the high metabolism of steroid hormones in high producing dairy cows, six studies were carried out, which in this thesis are divided in three chapters, involving hormone supplementation in lactating dairy cows. The first study aimed to increase the synchronization rate of dairy cows submitted to a fixed time artificial insemination (FTAI) estradiol (E2)/progesterone (P4)-based protocol. For this purpose, two experiments were performed, the first (n = 44 cows) compared a 2.0 vs 3.0 mg of estradiol benzoate (EB) associated to a P4 implant at the beginning of the protocol. The second experiment (n = 82 cows) performed presynchronization with GnRH prior to the onset of a FTAI protocol to produced different follicular development stages at the time of E2/P4: emergence vs. dominance. Daily ultrasound and hormone evaluations were performed. Other four experiments are described in the second (n = 1070 cows) and third (n = 1498 cows) chapter, which have been developed to evaluate the effect of P4 supplementation after ovulation in lactating dairy cows. In general, these studies evaluated the effect of supplementation on the corpus luteum (CL) development and function, mRNA abundance for interferon stimulated genes (ISG), on fertility of cows subjected to AI after estrus detection or FTAI protocol, or to embryo transfer. Increasing the EB dose from 2.0 for 3.0 mg did not improve emergence wave synchronization. In fact, it induced luteolysis in a larger number of cows. Altering the stage of the estrous cycle of the cows at the beginning of the E2/P4-based FTAI protocol did not improve synchronization of wave emergence. Post ovulation P4 supplementation did not affect CL development and function, and did not increase the mRNA abundance for ISG. Cows subjected to AI after estrus detection or after an E2/P4-based FTAI protocol did not have increased fertility. However when P4-supplemented cows were subjected to a GnRH-based FTAI protocol there was an improvement in the fertility of about 8%. Thus, we can concluded that regardless of the EB dose or stage of the estrous cycle at beginning of the E2/P4-based FTAI protocol, still there are cows that fail to have a synchronized emergence of a new wave and/or to ovulate at the end protocol. Additionally, depending on the protocol used, P4 supplementation may increase the fertility of dairy cows, but compromises the fertility when embryos are transferred.

Keywords: Artificial insemination; Embryo transfer; Estradiol; Progesterone; Synchronization

1 INTRODUCTION

In dairy cattle, fertility is critical to improve the efficiency of dairy production. It was observed that, in high producing herds (12,500 kg in 305 days of lactation), by reducing days open from 161 to 98, there was an increase in milk production of 1.51 kg/day/cow (RIBEIRO et al., 2012). On the last 50 years, it was observed a marked increase in the average of milk production associated to a decrease of fertility in dairy cattle (LUCY, 2001; WASHBURN et al., 2002). In the United States, in the late 70's, when average milk production was ~17 kg/day/cow, there were approximately two services per conception, whereas in 1999, almost three services were needed per conception, and milk production was greater than 25 kg/day/cow (WASHBURN et al., 2002). Increase of incidence of clinical and subclinical diseases (RIBEIRO et al., 2013), increased steroid metabolism associated with increase of dry matter intake (DMI) (PARR et al., 2002) and heat stress affect reproductive efficiency (SARTORI; BASTOS; WILTBANK, 2010).

High milk production is associated with increase of DMI (HARRISON et al., 1990) and liver blood flow, consequently, to the elevated steroid hormone metabolism (PARR et al., 1993; SANGSRITAVONG et al., 2002). Lactating dairy cows had bigger ovulatory follicles and corpora lutea (CL), nevertheless, they had lower plasma concentration of estradiol-17 β (E2) and progesterone (P4) than dairy heifers (SARTORI et al., 2004). Cows with higher milk production have lower circulating E2 at estrus, lesser duration of estrus, less events and standing times of estrus, despite having greater ovulatory follicles (LOPEZ et al., 2004) and multiple ovulation rate (WILTBANK et al., 2006). It has been shown also that cows ovulating larger follicle had lower pregnancy per artificial insemination (P/AI) (PEREIRA et al., 2014).

For improvement of reproductive performance of dairy cows, by using two GnRH doses and a single Prostaglandin F_{2 α} (PGF) treatment, Pursley et al. (1995) created the first fixed time artificial insemination (FTAI) protocol known as Ovsynch (GnRH – 7 d – PGF – 2 d – GnRH – 1 d - AI) . Nevertheless, to obtain higher P/AI at the Ovsynch, cows should be treated with the first GnRH between d 5 and d 13 of the estrous cycle (VASCONCELOS et al., 1999). This strategy resulted in greater synchronization of wave emergence, presence of CL during the protocol and greater ovulation rate. Although E2/P4-based protocols (BO et al., 1993) have limitations due to prohibition in some countries, they have been effectively used for FTAI in other countries (SOUZA et al., 2009; VASCONCELOS et al., 2011; PEREIRA et al., 2013, 2014). Despite been widely used, P4/E2-based FTAI protocols also present a relatively high

incidence of ovulation failure, and not optimized P/AI (around 30%) (SOUZA et al., 2009), requiring more studies to understand the cause of these failures in dairy cows. Currently, a 2.0 mg dose of estradiol benzoate (EB) at the onset of the protocol has been used in Nelore (*Bos indicus*) cattle with good results (MENEGETTI et al., 2009; PERES et al., 2009; SA FILHO et al., 2009). Nevertheless, it has been shown that after treatment with EB, nonlactating Holstein cows presented about half the circulating E2 in relation to nonlactating Nelore cows independent of EB dose (1.0, 2.0 or 4.0 mg) (BASTOS et al., 2011). Therefore, it is likely that the 2.0 mg dose of EB may be insufficient to provide an optimized wave emergence synchronization, especially for lactating dairy cows, which have even higher E2 clearance than nonlactating Holsteins (WILTBANK et al., 2006).

For improving the response to the FTAI protocols a presynchronization strategy has been proposed (MOREIRA et al., 2001; SOUZA et al., 2008). Although this concept is well defined in the GnRH-based protocols, few studies have evaluated the use of presynchronization protocols in dairy cows when using E2/P4-based FTAI protocols. Presynchronization-based protocols were used to increase the synchronization rate of wave emergence to the Ovsynch protocol ensuring greater P/AI (MOREIRA et al., 2001). Not only the phase of the estrous cycle is important for obtaining higher fertility (VASCONCELOS et al., 1999), but it is also necessary to consider that high concentration of P4 during emergence and development of the future ovulatory follicle increases the fertility of dairy cows (WILTBANK et al., 2006; BISINOTTO et al., 2013). Another presynchronization protocol, called Double-Ovsynch, was developed with positive and consistent results (SOUZA et al., 2008). It has been shown that high circulating P4 both before and after AI is associated with increased fertility (WILTBANK et al., 2006). Thus, supplemental P4 has been used to increase fertility in dairy cows before (BISINOTTO et al., 2013) and after AI (WILTBANK et al., 1956; JOHNSON; ROSS; FOURT, 1958; LARSON; BUTLER; CURRIE, 2007).

In ruminants, circulating P4 affects the LH release profile (ROBINSON et al., 2000). Moreover, LH receptors presence in the CL (FITZ et al., 1982) suggests LH involvement on CL developmental and function during metestrus and diestrus. *In vitro*, luteal cells increased P4 production, when challenged with human chorionic gonadotropin (hCG; similar molecule to the LH; FITZ et al., 1982). Studies have shown that P4 supplementation after ovulation has benefits for embryo development (CLEMENTE et al., 2009; O'HARA et al., 2014). However, fertility data are scarce on the effects of P4 supplementation in recipient cows prior to a single embryo transfer.

High concentration of circulating P4 on metestrus or early diestrus has been associated with advancement of conceptus elongation (CLEMENTE et al., 2009; O'HARA et al., 2014), an associated increase in interferon- τ (IFN) (MANN; FRAY; LAMMING, 2006). The IFN is the signal for maternal recognition of pregnancy in ruminants associated with prevention of luteolysis. This signal ensures continued production of P4 by the CL (PLANTE; HANSEN; THATCHER, 1988). During early pregnancy, the presence of the conceptus in the uterus suppressed the expression of both oxytocin and E2 receptors in the uterus (ROBINSON et al., 2001). The absence of oxytocin receptor in the endometrium prevents the release of luteolytic pulses of PGF, thereby sustaining lifespan of the CL and P4 production (DORNIK; SPENCER, 2013). In addition to preventing luteolysis, IFN also induces expression of a large number of IFN stimulated genes (ISGs) in the endometrium (HICKS et al., 2003; GIFFORD et al., 2008). Not only endometrial cells express ISGs, but also other tissues, such as peripheral blood mononuclear cells (PBMC). Pregnant cows have shown increased ISG expression than nonpregnant cows (MONTEIRO et al., 2014; RIBEIRO et al., 2014). ISGs, such as 2',5'-oligoadenylate synthetase, myxovirus resistance 1/interferon-inducible p78 (MX1), myxovirus resistance 2 (MX2), interferon-stimulated gene 15 (ISG15), receptor transporter protein 4 (RTP4) were found expressed in PBMC showing difference on the expression between pregnant and nonpregnant ewes (YANKEY et al., 2001; OLIVEIRA et al., 2007; GIFFORD et al., 2008;) and cows (MONTEIRO et al., 2014; RIBEIRO et al., 2014).

1.1 Hypothesis

1.1.1 The increase in the dose of EB from 2.0 mg for 3.0 mg would increase synchronization of follicular wave emergence and thereby increase synchronization during the protocol;

1.1.2 Presynchronization with a single GnRH treatment either 3 or 7 days before EB, would synchronize the stage of the follicular wave at the start of the E2/P4 protocol and produce a greater synchronization rate to the E2/P4 protocol;

1.1.3 P4 supplementation 3 days after FTAI would not alter the CL development and function;

1.1.4 P4 supplementation post ovulation would stimulate mRNA abundance for ISG in PBMC;

1.1.5 P4 Supplementation post ovulation would increase fertility of lactating dairy cows subjected to AI by estrus detection, to FTAI, and to embryo transfer.

1.2 Objectives

1.2.1 To evaluate whether 3.0 mg of EB increases the proportion of cows synchronized to E2/P4-based FTAI protocol;

1.2.2 To evaluate whether pre-synchronization with GnRH, 3 or 7 days before starting FTAI protocol, improves synchronization rate of lactating dairy cows subjected to a E2/P4-based FTAI protocol;

1.2.3 To observe the effect of the supplementation with P4, in lactating dairy cows, 3 days after FTAI, on the CL formation, and on the P4 plasma concentration throughout the estrous cycle;

1.2.4 To evaluate the mRNA abundance for ISG in cows subjected to P4 supplementation after AI by estrus detection, and FTAI;

1.2.5 To evaluate the fertility of lactating dairy cows supplemented with P4 subjected to AI by estrus detection, to FTAI, and embryo transfer.

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2 EVALUATION OF ESTRADIOL-BASED PROTOCOLS FOR TIMED ARTIFICIAL INSEMINATION IN DAIRY CATTLE: INCREASING ESTRADIOL BENZOATE, PRESYNCHRONIZATION WITH GnRH, AND IMPEDIMENTS FOR A SUCCESSFUL PROTOCOL

Abstract

With the objective to optimize fixed time artificial insemination (FTAI) protocols based on estradiol and progesterone (P4), two experiments were performed using synchronization with increased estradiol benzoate (EB; 2 [EB2] vs 3mg [EB3]; Expt 1; n = 44) or using presynchronization with a single GnRH treatment 3 or 7 d before initiation of the protocol (Expt 2; n = 82). In both experiments all cows had ovulation synchronized. All cows were treated with EB at the time of introduction of an intravaginal P4 implant (d 0). On d 7, cows were given 25mg prostaglandin F_{2α} and on d 8, the P4 implant was removed and cows given 1mg estradiol cypionate to induce ovulation. All cows received FTAI on d 10. In both Expts, daily ultrasound evaluations (US) were performed during the protocol and circulating P4 was evaluated during the protocol in Expt 2. Pregnancy/AI (P/AI) was determined on d 31 and d 59 after FTAI. Statistical analyses were performed with Mixed, Glimmix, and Freq procedures of SAS. In Expt 1, EB dose did not change time to wave emergence, but EB3 compared to EB2 decreased percentage of cows with CL on d 7 (19.8 vs. 55.3%) and time to ovulation (10.4 vs. 10.9 d). In Expt 2, ovulation to GnRH given 3 or 7 d before initiating the protocol (d 0) did not alter P/AI but did reduce the percentage of cows without a CL on d 0, percentage that regressed the CL (d 0 to d 7), and the size of the ovulatory follicle. Regardless of treatment, more cows on d 10, with complete (P4 < 0.1 ng/mL) compared to partial (P4 ≥ 0.1 and < 0.22 ng/mL) CL regression ovulated to the protocol (81.2 vs. 58.0%), and had increased P/AI (47.4 vs. 21.4%). An analysis of data from both Expts showed that only 73.8% (93/126) of cows had synchronized wave emergence between D1 and D6 and only 77.8% (98/126) of cows ovulated at the end of the protocol. As expected, fertility was much greater in synchronized cows (P/AI 61.3% [46/75]) compared to cows not optimally synchronized (15.7% [8/51]). Thus, although current FTAI protocols using EB and P4 produce satisfactory results for lactating dairy cows, there is still room for improvement since less than 60% (75/126) of the cows were correctly synchronized. Presynchronization with GnRH or increasing the dose of EB to 3mg were not effective in increasing the synchronization rate.

Keywords: Fixed time AI; Estradiol benzoate; Progesterone; Synchronization

2.1 Introduction

Reproductive efficiency in dairy herds around the world has declined for a number of decades (LUCY, 2001; MEE, 2012), although recent data on daughter pregnancy rate in the USA has indicated a stabilization in genotypic values and a dramatic increase in phenotypic values for reproduction (NORMAN et al., 2009; WILTBANK; PURSLEY, 2014). The improvement in reproductive performance can be partially explained by the use of systematic

reproductive management programs in the USA including programs for synchronization of ovulation and fixed time AI (FTAI). These programs allow cows to be inseminated at a predesignated time without the need for detection of estrus and thus increase the AI submission rate (WILTBANK et al., 2014). Two types of hormonal programs have been utilized for synchronization of ovulation. Programs such as Ovsynch, use GnRH and prostaglandin $F_{2\alpha}$ (PGF) to synchronize ovulation (PURSLEY; MEE; WILTBANK; 1995; PURSLEY; KOSOROK; WILTBANK, 1997), whereas programs in many parts of the world combine estradiol (E2), progesterone (P4), and PGF to synchronize ovulation (BO et al., 1995b; SOUZA et al., 2009; BARUSELLI et al., 2012). Both types of FTAI programs attempt to synchronize follicular waves, corpus luteum (CL) function, and circulating reproductive hormones in order to allow ovulation of an optimal-sized follicle in an optimal hormonal environment at a preappointed time.

In GnRH-based programs it is well known that many cows are not correctly synchronized by the program. For example, Giordano et al. (2012) reported that only about 50% of cows treated with Ovsynch were correctly synchronized, whereas Double-Ovsynch resulted in about 70% synchronization. To date, synchronization rates during E2/P4-based programs in lactating dairy cows have not been adequately evaluated. Early studies that established the physiological basis for these programs utilized beef heifers and found that treatment with various forms of E2 led to suppression of gonadotropins, regression of growing follicles, and emergence of a new follicular wave about 4 d after E2 treatment (BO et al., 1993, 1994, 1995). Souza et al. (2009) evaluated 45 lactating dairy cows using daily ultrasound evaluations and reported that 84.4% (38/45) had synchronized emergence of a follicular wave after treatment with 2 mg E2-benzoate (EB) combined with insertion of controlled internal drug release containing P4. In addition, they found that 83.3% of cows had synchronized ovulation at the end of the protocol (Souza et al., 2009). Thus, similar to GnRH-based programs, protocols using E2/P4 may have problems with correct synchronization of the follicular wave at the beginning of the program and synchronized ovulation at the end of the program. Critical features of FTAI protocols that need to be optimized include: follicular wave emergence and growth (WILTBANK et al., 2011), concentration of P4 during follicular growth (BISINOTTO et al., 2013; WILTBANK et al., 2014), complete lysis of the CL at the time of AI (SOUZA et al., 2007; PEREIRA et al., 2013b; WILTBANK et al., 2014), and size of the ovulatory follicle (WILTBANK et al., 2011; PEREIRA et al., 2013a, 2014).

Programs that use E2/P4 to synchronize ovulation have generally used 2 mg of EB at the start to synchronize the follicular wave, although the early studies used 5 mg of various E2

derivatives, sometimes combined with P4 (BO et al., 1993, 1994, 1995a). The studies of Souza et al. (2009) reported that higher-producing lactating dairy cows had earlier follicular wave emergence than lower producing dairy cows after treatment with 2 mg EB. They postulated that the high E2 metabolism that has been found in lactating dairy cows (SANGSRITAVONG et al., 2002; WILTBANK et al., 2006) may be responsible for a more rapid decrease in circulating E2 concentrations after EB treatment. Thus, high-producing lactating dairy cows may require a greater dose of EB to achieve follicular wave regulation than is required in lower-producing cows or beef cattle.

The GnRH-based FTAI programs have better fertility when initiated on certain days of the estrous cycle (VASCONCELOS et al., 1999; MOREIRA et al., 2000), such as d 6 or 7 when there is a high ovulation rate in response to the first GnRH treatment and high circulating P4 during the protocol. Presynchronization programs have been developed to increase the percentage of cows at these optimal stages of the estrous cycle such as Presynch-12-Ovsynch (MOREIRA et al., 2001; PORTALUPPI; STEVENSON, 2005), Double-Ovsynch (SOUZA et al., 2008; HERLIHY et al., 2012), or G-6-G (BELLO; STEIBEL; PURSLEY, 2006; WILTBANK; PURSLEY, 2014). Optimal times of the estrous cycle to initiate E2-P4 protocols or effects of presynchronization strategies on the efficacy of E2-P4 protocols have not yet been reported.

Thus, these studies were based on three hypotheses related to E2/P4 protocols. First, we hypothesized that an increased dose of EB would increase synchronization of follicular wave emergence and thereby increase synchronization during the protocol. Second, we hypothesized that presynchronization with a single GnRH treatment would synchronize the stage of the follicular wave at the start of the E2/P4 protocol and produce a greater synchronization rate to the E2/P4 protocol. Finally, we hypothesized that complete characterization of the physiological response to E2/P4 protocols would demonstrate that synchronization rate following the protocol was not optimal and was due to specific physiological responses to the protocol. Two experiments were performed with the following objectives. The first experiment evaluated whether 3.0 mg of EB produced better synchronization of follicular wave emergence than 2.0 mg. The second experiment evaluated whether pre-synchronization with GnRH, 3 or 7 d before starting the protocol, would improve the synchronization rate of the cows. Finally, the results from both experiments were combined to evaluate specific physiological reasons for lack of synchronization to the protocol.

2.2 Materials and methods

The Animal Research Ethics Committee of “Luiz de Queiroz” College of Agriculture (ESALQ)/ University of São Paulo approved all procedures involving cows in this study.

2.2.1 Experiment 1

Cows, housing and diets

This experiment was conducted in the dairy farm of ESALQ in São Paulo state, Brazil and used 20 primiparous and 24 multiparous lactating Holstein cows. At the beginning of the study, cows averaged (\pm SEM) 23.8 ± 1.5 (range, 9.5 to 41.5) kg of milk/d, 350.8 ± 36.0 (range, 63 to 791) DIM, with body condition score (BCS) of 2.8 ± 0.1 (range, 2.25 to 3.25, using a 1 [emaciated] to 5 [obese] scale; [ELANCO, 2009]) and age of 3.9 ± 0.1 yr (range, 3.0 to 4.8). During the experimental period, cows were housed in a tie stall barn equipped with sprinklers and fans. Cows were milked two times daily, and milk yield for each cow was recorded. Feed was provided two times daily, concurrent with milking. Cows were fed *ad libitum* a TMR-based diet of corn silage, with corn-soybean meal-based concentrate and minerals and vitamins, which was balanced to meet or exceed the nutritional requirements of lactating dairy cows (NATIONAL RESEARCH COUNCIL - NRC, 2001).

Protocols and treatments

The cows were randomized to receive either 2.0 mg (EB2; n = 23) or 3.0 mg (EB3; n = 21) of EB (Sincrodiol, Ourofino, Ribeirão Preto, SP, Brazil) at the start of a FTAI protocol (Figure 1) that included insertion of an intravaginal implant containing 1.0 g of P4 (Sincrogest, Ourofino) at same time as EB treatment (d 0), PGF treatment on d 7 (33.5 mg of dinoprost trometamine; Lutalyse, Zoetis, São Paulo, SP, Brazil) and removal of the P4 implant on d 8 together with treatment with 1.0 mg of estradiol cypionate (ECP, Zoetis, São Paulo, SP, Brazil). Two d after ECP treatment (d 10), all of the cows were inseminated with commercial semen from bulls with proven fertility (Figure 2.1).

Ultrasonography evaluation.

During the protocol, ovaries of all cows were examined by transrectal ultrasonography (DP-2200, Mindray, Huntingdon, UK, PE29 6SZ) using a 7.5 MHz linear-array transducer, every 24 h from d 0 until ovulation at the end of the protocol (or d 12) and on d 17. All CL and

follicles ≥ 4 mm were measured and mapped. These daily ovarian maps were used to determine: day of emergence of follicular wave, day of follicular deviation, size of follicle at time of deviation of the future ovulatory follicle, day of ovulation, number of ovulations, size of ovulatory follicle, and presence and size of CL on each d. On d 17, ovulation was confirmed by detection of a CL using ultrasonography. Pregnancy diagnoses were performed at 31 and 59 d after FTAI using ultrasonography.

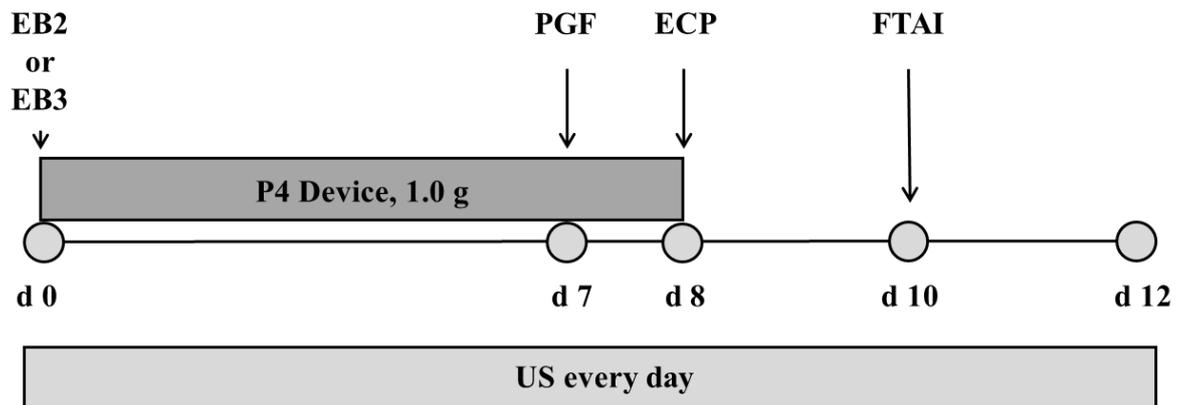


Figure 2.1 – Diagram of activities for experiment 1. Study d 0 is the day the protocol began with insertion of intravaginal P4 implant and estradiol benzoate (**EB**) treatment with either 2 mg of EB (**EB2**; n = 23) or 3 of mg EB (**EB3**; n = 21). On d 7, 33.5 mg of dinoprost trometamine (**PGF**) was administrated and on d 8 the P4 implant was removed and 1.0 mg of estradiol cypionate (**ECP**) was administrated. On d 10, cows received fixed time AI (**FTAI**). Transrectal ultrasonography (**US**) was performed day from d 0 to ovulation (or d 12)

2.2.2 Experiment 2

Cows, housing and diets.

This experiment was conducted on a dairy farm in São Paulo State, Brazil using 82 lactating dairy cows (Holstein, n = 70; Holstein x Jersey crossbred, n = 12), primiparous (n = 37) and multiparous (n = 45). At the beginning of the study, cows averaged (\pm SEM) 33.8 ± 1.0 (range, 19.0 to 53.8) kg of milk/d, 161.3 ± 18.4 (range, 39 to 359) DIM, with BCS of 2.7 ± 0.1 (range, 2.25 to 3.50) and age of 4.7 ± 0.2 yr (range, 2.2 to 10.2). During the experimental period, cows were housed in a free stall barn equipped with sprinklers and fans. Cows were milked three times daily, and milk yield for each cow was recorded automatically. Feed was provided three times daily, concurrent with milking. Cows were fed *ad libitum* a TMR-based diet of corn silage, barley, and corn and soybean meal-based concentrate with minerals and vitamins, which was balanced to meet or exceed the nutritional requirements of lactating dairy cows (NRC, 2001).

Protocols and treatments.

The cows were subjected to the same FTAI protocol as used in experiment 1, except that all cows received 2 mg of EB on d 0 (Figure 2.2). Prior to the FTAI protocol cows were randomly assigned to receive GnRH (10.5 µg busserelin acetate; Sincroforte, Ourofino) either: 7 d before the start of the FTAI protocol (n = 40) or 3 d before the start of the FTAI protocol (n = 42). Three groups were designated following determination of ovulatory response to the GnRH treatment: Cows that ovulated to the d 7 GnRH treatment (G7), cows that ovulated to the d 3 GnRH treatment (G3), and cows that did not ovulate following GnRH treatment (NoOv).

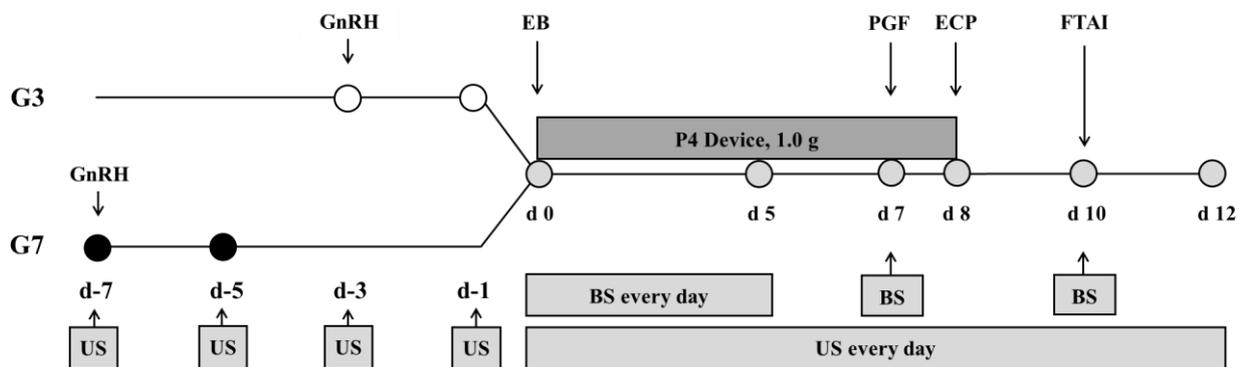


Figure 2.2 – Diagram of activities for experiment 2. Prior to d 0 (Initiation of protocol) cows received GnRH (10.5 mg busserelin acetate) on either d -7 (**G7**; n = 40) or d -3 (**G3**; n = 42). All cows received the same synchronization protocol (d 0 – P4 implant + 2 mg of estradiol benzoate [**EB**]; d 7 – 33.5 mg of dinoprost trometamine [**PGF**]; d 8 – P4 implant removed + 1.0 estradiol cypionate [**ECP**]; d 10 – fixed time AI [FTAI]). Transrectal ultrasonography (**US**) was performed on d -7 and -5, for cows of G7 group, and on d -3 and -1, for cows of G3 group and subsequently on a daily basis in all cows from d 0 to ovulation (or d 12). Blood sample (**BS**) were collected daily from d 0 to 5 and on d 7 and d 10

Ultrasonography evaluation.

During the protocol, ovaries of all cows were examined by transrectal ultrasonography (DP-2200, Mindray, Huntingdon, UK, PE29 6SZ) using a 7.5 MHz linear-array transducer, on d -7 and d -5 in the cows treated with GnRH on d 7; on d -3 and d -1 in the cows treated with GnRH on d 3; and on all cows on a daily basis from d 0 until ovulation after the end of the protocol (or d 12) and on d 17 (to confirm ovulation). All CL and follicles ≥ 4 mm were measured and mapped and maps were used for the same determinations described for experiment 1. Pregnancy diagnoses were performed at 31 and 59 d after FTAI.

Cows were considered positive for emergence of a new wave when emergence occurred between d 1 and d 6. Cows that had the ovulatory follicular wave emerge before d 1 were classified as lacking emergence of a new wave and if ovulation occurred from this follicular wave were classified as ovulating a persistent follicle.

Blood sampling and analysis of progesterone in plasma.

Blood was sampled from all 82 cows every 24 h from d 0 until d 5, and on d 7 and d 10, by puncture of the coccygeal vein or artery into evacuated tubes containing Sodium Heparin (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). Immediately after collection, tubes with blood were placed on ice and kept refrigerated until transported to the laboratory within 4 to 5 h after collection. Blood tubes were centrifuged at 1,700 x g for 15 min at 4°C for plasma separation. Aliquots of plasma were frozen at -20°C until assayed. Concentrations of P4 were analyzed by RIA using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). The sensitivity of the kit was 0.02 ng/mL. The intra-assay CVs were 5.4% in assay 1 and 4.3% in assay 2. The inter-assay CV was 4.1%.

2.2.3 Analysis of synchronization using cows from both experiments

Cows in both studies were treated with a similar EB/P4-based FTAI protocol and had daily ultrasound evaluations during the protocol. Therefore, an analysis was performed to determine the reasons that cows failed to synchronize during the protocol. In this analysis, data from all 126 cows from both experiments were evaluated (n = 44 from Experiment 1; n = 82 from Experiment 2). Cows that had emergence of a new follicular wave after EB treatment and ovulated at the end of the protocol were considered synchronized.

2.2.4 Statistical Analysis

Both experiments the sample size was calculated using the POWER procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC) using a one-sided test to provide sufficient experimental units to detect statistical significance ($\alpha = 0.05$; $\beta = 0.20$) when day of emergence new wave change from 3 for 4. For this were necessary approximately 14 cows per treatments.

Continuous data with repeated measures over time were analyzed by ANOVA using the MIXED procedure of SAS version 9.3 with models fitting a Gaussian distribution. Data were tested for normality of residuals, and data with residuals not normally distributed were transformed before analysis. The models included the fixed effects of treatment, day of measurement, parity, breed, BCS, milk yield categorized within parity during the week of d 0

as above or below the mean value, interactions between treatment and day and treatment and parity, and the random effects of cows nested within treatment. The covariance structure that resulted in the smallest Akaike's information criterion was selected for the model. Model fitting was evaluated using the fit statistics.

Categorical data were analyzed by logistic regression using the GLIMMIX procedure of SAS fitting a binary distribution. The models included the fixed effects of treatment, parity, breed, BCS category, and milk yield categorized within parity on the week of d 0 as above or below the mean value. The Kenward-Roger method was used to calculate the denominator degrees of freedom to approximate the F tests in the mixed models. Model fitting was evaluated using the fit statistics. The estimates were back-transformed using the ILINK function of SAS to generate the adjusted percentages. The FREQ procedure of SAS was used for the analyses of the combined data from experiment 1 and 2 or for analyses in experiment 2 when a treatment outcome was 0 or 100%. In all analyses, differences were considered significant when $P < 0.05$, whereas tendencies were considered when $0.15 > P \geq 0.05$.

2.3 Results

2.3.1 Experiment 1

Day of emergence, day of deviation, and size of future ovulatory follicle at time of deviation were not affected by EB dose (Table 2.1). The percentage of cows with a CL on d 7 was reduced ($P < 0.05$) for cows receiving 3 mg of EB compared to 2 mg. The percentage of cows ovulating at the end of the protocol was high (~90%) and not altered by EB treatment. However, time of ovulation was later ($P < 0.01$) for cows that received 2 mg EB compared to 3 mg EB. In addition, multiple ovulation was surprisingly high (33%) but not affected by treatment. There was also no effect of treatment on P/AI (Table 2.1).

Absence of CL on d 0 affected ($P < 0.01$) number of follicles ovulating per cow at the end of the protocol, with 2.1 ± 0.21 ovulations for cows without a CL and 1.2 ± 0.08 for cows with a CL. Similarly, percentage of cows with multiple ovulation on d 10 tended ($P = 0.06$) to be greater in cows without a CL ($80.2\% \pm 19.0$ [4/5]) than cows with a CL ($26.9\% \pm 8.6$ [9/34]). In contrast, there was no effect of absence of a CL on d 7 on multiple ovulation rate ($29.0\% \pm 9.5$ [7/24] vs. $39.9\% \pm 13.0$ [6/15]).

Table 2.1 – Results (LSM \pm SEM) for experiment 1, comparing 2.0 (n = 23) vs. 3.0 (n = 21) mg of estradiol benzoate (EB) at the beginning of an E2/P4-based FTAI protocol (d 0)

	EB2	EB3	P
Day of emergence ¹ , d	3.4 \pm 0.17	3.6 \pm 0.19	0.36
Synchronized wave/total ² , %	82.6 \pm 7.9 (19/23)	71.4 \pm 9.9 (15/21)	0.39
Size of future ovulatory follicle at deviation ³ , mm	8.0 \pm 0.29	8.0 \pm 0.30	0.84
CL presence on d 7, %	55.3 \pm 13.3 (13/21)	19.8 \pm 9.8 (4/17)	< 0.05
Day of deviation ³ , d	5.9 \pm 0.21	6.1 \pm 0.23	0.63
Maximum size of ovulatory follicle ³ , mm	14.1 \pm 0.68	13.9 \pm 0.62	0.80
Day of ovulation, d	10.9 \pm 0.14	10.4 \pm 0.13	< 0.01
Ovulation rate ⁴ , %	89.1 \pm 6.9 (20/23)	92.2 \pm 5.9 (19/21)	0.71
Multiple ovulation rate ⁵ , %	26.5 \pm 11.0 (5/20)	42.1 \pm 12.1 (8/19)	0.35
Synchronized wave emergence and single ovulation, %	56.6 \pm 11.0 (13/23)	33.4 \pm 10.9 (7/21)	0.16
P/AI			
31 d, %	47.7 \pm 17.2 (11/23)	40.9 \pm 15.0 (9/21)	0.71
59 d, %	42.6 \pm 16.5 (10/23)	36.7 \pm 14.2 (8/21)	0.73

¹ Only cows that had emergence of a new wave between d 1 and d 6 were included

² Number of cows that had emergence a new wave between d 1 and d 6 divided by the total number of cows treated

³ Only cows that had new wave emergence (d 1 to d 6) and a single-ovulation were included

⁴ Number of cows that ovulated divided by number of cows treated

⁵ Number of cows that ovulated two or more follicles divided by the number of cows treated

2.3.2 Experiment 2

Days of events and size of ovarian structures.

Day of emergence and day of deviation were delayed in cows in G7 compared with G3 and NoOv (Table 2.2). There was no difference among groups for percentage of cows with synchronized wave emergence and size of the future ovulatory follicle at deviation. A greater percentage of NoOv cows had no CL present at the time of protocol initiation compared to the other two groups. In addition, a greater percentage of these cows had CL regression between d 0 and d 3 compared to G7 or G3. However, there were no differences between groups in CL regression between d 3 and 7 (Table 2.2). Overall, a greater ($P < 0.05$) percentage of cows regressed the CL between d 0 and 7 in the NoOv group compared to the G3 and G7 groups. This may have influenced size of the ovulatory follicle which was greater for cows in the NoOv

group compared to G3 and G7 groups (Table 2.2). Independent of treatment group, cows that had a CL on d 7 ovulated a smaller ($P = 0.05$) follicle (14.9 ± 0.3 mm) than cows that did not have a CL on d 7 (16.5 ± 0.5 mm).

A comparison was made between milk production and various measures of timing and size of the future ovulatory follicles, which includes only those cows with emergence of a new follicular wave (d 1 to d 6) and single ovulation (data not shown). Day of follicle wave emergence was delayed ($P = 0.03$) in cows with lower milk yield (3.4 ± 0.20 d) compared to cows with higher milk yield (2.8 ± 0.20 d). However, milk yield did not affect the day of deviation, day of ovulation, size of future ovulatory follicle at deviation, or maximum size of ovulatory follicle. In addition, the percentage of cows that had emergence of a new follicular wave (d 1 to d 6) or percentage of cows with multiple ovulation was not affected by milk yield.

In Figure 2.3 is shown the dynamics of ovulatory follicle growth for cows that ovulated a persistent follicle (ovulated a single follicle that was present on d 0; $n = 17$) compared to cows that ovulated a single follicle that emerged from a new follicular wave (d 1 to d 6 of protocol; $n = 42$). On all days during the protocol, the future ovulatory follicle was larger for cows that ovulated a persistent follicle than cows that ovulated a follicle that emerged during the protocol ($P < 0.05$). Cows ovulating a new follicle tended ($P \leq 0.07$) to have higher P/AI (Figure 2.3).

Concentration of progesterone.

At the time of protocol initiation (d 0) circulating P4 concentrations were greater in G7 and NoOv cows compared to G3 cows (Figure 2.4). As the protocol progressed, the P4 concentrations progressively decreased in NoOv, as CL continued to regress, and increased in G3, with growth of the new CL. Therefore, concentrations of P4 were greater in G7 and G3 than in NoOv cows on d 4, d 5, and d 7 of the protocol. By d 7, circulating P4 concentrations had decreased ($P < 0.05$) in G7 and NoOv cows compared to d 0 values but had not changed in G3 cows. A comparison of overall circulating P4 concentrations during the entire period indicated that there was a tendency for a treatment effect ($P = 0.06$), an effect of day ($P < 0.001$), and a treatment by day interaction ($P = 0.01$). Concentrations of P4 were 0.5 ng/mL greater in G7 (3.2 ± 0.3) compared to NoOv (2.7 ± 0.2) cows, with G3 (2.7 ± 0.3) cows not significantly different from the other two groups.

Independent of treatment group, cows that were pregnant on d 31 or d 59 after FTAI had greater circulating P4 concentrations during the protocol than cows that were not pregnant ($P = 0.01$; Figure 2.5). There was also an effect of day ($P < 0.001$), but no day by pregnancy status interaction. For all days that were analyzed during the protocol, except d 7, cows that

were later found to be pregnant had greater circulating P4 compared to cows that were subsequently found to be non-pregnant.

On the day of FTAI, the circulating P4 concentrations were categorized in order to evaluate whether individual cows had undergone complete luteolysis ($P4 < 0.10$ ng/mL) or partial luteolysis ($P4 = 0.10$ to 0.22 ng/mL). The percentage of cows that ovulated at the end of protocol was greater ($P = 0.04$) for cows with complete compared to partial luteolysis (Figure 2.6). In addition, there was an effect of complete luteolysis on P/AI at either d 31 ($P = 0.03$) and d 59 ($P = 0.05$; Figure 2.6). When only cows that ovulated were considered in the analysis, luteolysis status tended ($P = 0.08$) to influence P/AI on d 31 and d 59 ($P = 0.14$) after FTAI (Figure 2.6).

Ovulation rate and pregnancy per AI.

Day of ovulation and multiple ovulation rate were not different among groups, but ovulation rate tended to be higher on NoOv than G7, and G3 did not differ from the other treatments (Table 2.2). However, the percentage of cows that ovulated at the end of the protocol was not different ($P = 0.92$) for cows that had emergence of a new follicular wave (d 0 to d 6; $71.7 \pm 6.1\%$ [42/59]) compared to cows that did not have emergence of a new follicular wave ($72.9 \pm 9.7\%$ [17/23]).

The P/AI on d 31 and d 59 were not affected by treatment (Table 2.2). However, independent of treatment, cows that did not have a new wave emergence synchronized compared to cows with a new follicle tended to have a decrease in P/AI on d 31 ($20.6\% \pm 8.5$ [6/23] vs. $43.3\% \pm 7.0$ [27/59]; $P = 0.06$) and d 59 ($17.1\% \pm 7.7$ [5/23] vs. $37.9\% \pm 6.8$ [24/59]; $P = 0.07$) after FTAI.

Independent of treatment group, cows that had CL regression between d 0 and 3 compared to cows with no CL regression tended to have lower P/AI on d 31 ($23.5\% \pm 9.7$ [5/20] vs. $45.6\% \pm 8.2$ [23/45]; $P = 0.11$) and on d 59 ($18.1\% \pm 8.7$ [4/20] vs. $42.5\% \pm 8.3$ [22/45]; $P = 0.07$). In contrast, when all cows that had CL regression between d 0 and 7 were compared to cows that did not have CL regression, there was no difference in P/AI based on either the d 31 ($36.5\% \pm 9.0$ [14/33] vs. $40.1\% \pm 9.2$ [14/32]) or d 59 ($32.6\% \pm 8.9$ [13/33] vs. $36.2\% \pm 9.1$ [13/32]) pregnancy diagnosis.

Table 2.2 – Results (LSM ± SEM) for experiment 2, in which cows ovulated following treatment with 10.5 µg buserelin acetate (GnRH) 3 d (G3; n = 24) or 7 d (G7; n = 20) before the start of the protocol (d 0). Cows that did not ovulate to GnRH were allocated in another group (NoOv; n = 38)

	G3	G7	NoOv	P
Day of emergence ¹ , d	2.9 ± 0.25 ^y	3.6 ± 0.27 ^x	2.8 ± 0.21 ^y	0.08
Synchronized wave/total ² , %	78.7 ± 8.9 (17/24)	82.3 ± 8.7 (15/20)	78.3 ± 7.5 (27/38)	0.92
Size of future ovulatory follicle at deviation ³ , mm	8.2 ± 0.29	9.1 ± 0.38	8.6 ± 0.25	0.19
Day of deviation ³ , d	5.2 ± 0.38 ^b	6.4 ± 0.36 ^a	5.2 ± 0.25 ^b	< 0.01
Corpus luteum				
No CL on d 0, %	0.0 ^b (0/24)	0.0 ^b (0/20)	36.8 ^a (14/38)	< 0.01
Early regression (d 0 to 3) ⁴ , %	0.0 ^b (0/24)	10.0 ^b (2/20)	45.8 ^a (11/24)	0.01
Later regression (d 3 to 7) ⁴ , %	34.2 ± 10.7 (8/24)	21.1 ± 9.7 (4/20)	26.3 ± 9.8 (6/24)	0.63
Regression (d 0 to 7) ⁴ , %	35.6 ± 10.7 ^b (8/24)	30.5 ± 11.0 ^b (6/20)	72.5 ± 9.9 ^a (17/24)	0.02
Maximum size of ovulatory follicle ³ , mm	15.1 ± 0.75 ^b	14.6 ± 0.83 ^b	16.6 ± 0.57 ^a	0.04
Day of ovulation, d	10.6 ± 0.10	10.7 ± 0.13	10.6 ± 0.08	0.75
Ovulation rate ⁵ , %	71.2 ± 10.2 ^{ab} (18/24)	53.8 ± 11.8 ^b (11/20)	81.7 ± 6.4 ^a (30/38)	0.12
Multiple ovulation rate, %	19.7 ± 14.7 (3/18)	12.3 ± 14.0 (1/11)	8.5 ± 6.0 (3/30)	0.70
Synchronized wave emergence and single ovulation, %	42.6 ± 12.6 (11/24)	32.9 ± 12.8 (7/20)	44.7 ± 8.8 (17/38)	0.70
P/AI				
31 d, %	41.2 ± 11.5 (11/24)	35.6 ± 12.0 (7/20)	42.6 ± 9.1 (15/38)	0.89
59 d, %	38.2 ± 11.3 (10/24)	36.8 ± 12.1 (7/20)	34.1 ± 8.6 (12/38)	0.95

^{a,b} P value ≤ 0.05

^{x,y} P value > 0.05 and ≤ 0.10

¹ Only cows that had emergence of a new wave between d 1 and d 6 were included

² Number of cows that had emergence of a new wave between d 1 and d 6 divided by the total of cows treated

³ Only cows that had emergence of a new wave (d 1 to d 6) and single ovulation were included

⁴ Only cows that had a CL on d 0 were included in the analysis

⁵ Number of cows that ovulated divided by the number of cows treated

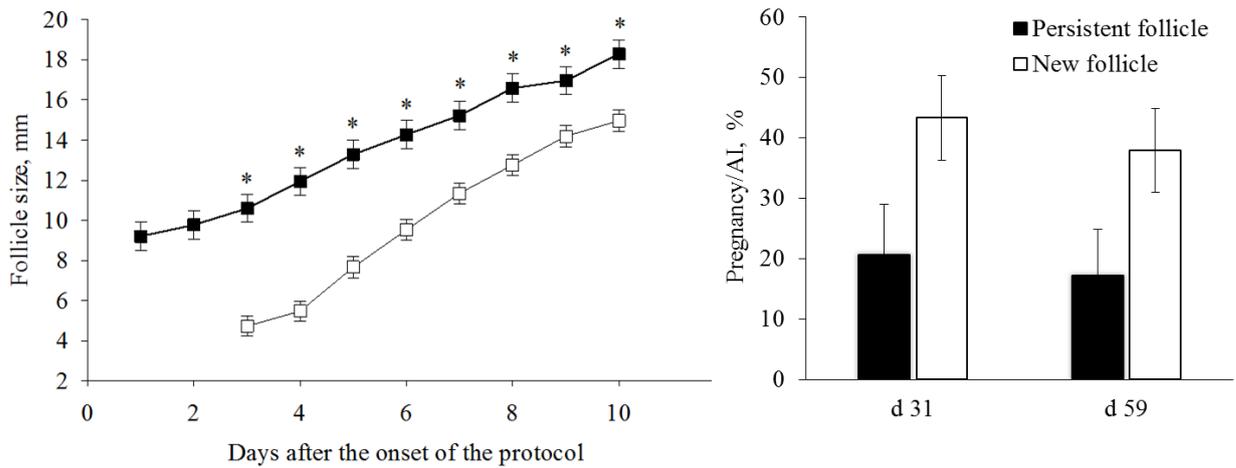


Figure 2.3 –Future ovulatory follicle size between d 1 and d 10 of protocol (left) and Pregnancy/AI (P/AI) of single-ovulating cows that ovulated a persistent or a new follicle (right) independent of treatment. Cows were considered to have ovulated a persistent follicle ($n = 17$) when they ovulated a follicle that was present on d 0. Cows ovulating a new follicle ($n = 42$) were considered when they had emergence of a new follicular wave between d 1 and d 6. There was an interaction between follicle type and day ($P < 0.001$). Cows that ovulated a persistent follicle tended to have a lower P/AI on d 31 ($P = 0.06$) and d 59 ($P = 0.07$) than those that ovulated a new follicle. *Within day, effect of follicle age ($P < 0.05$)

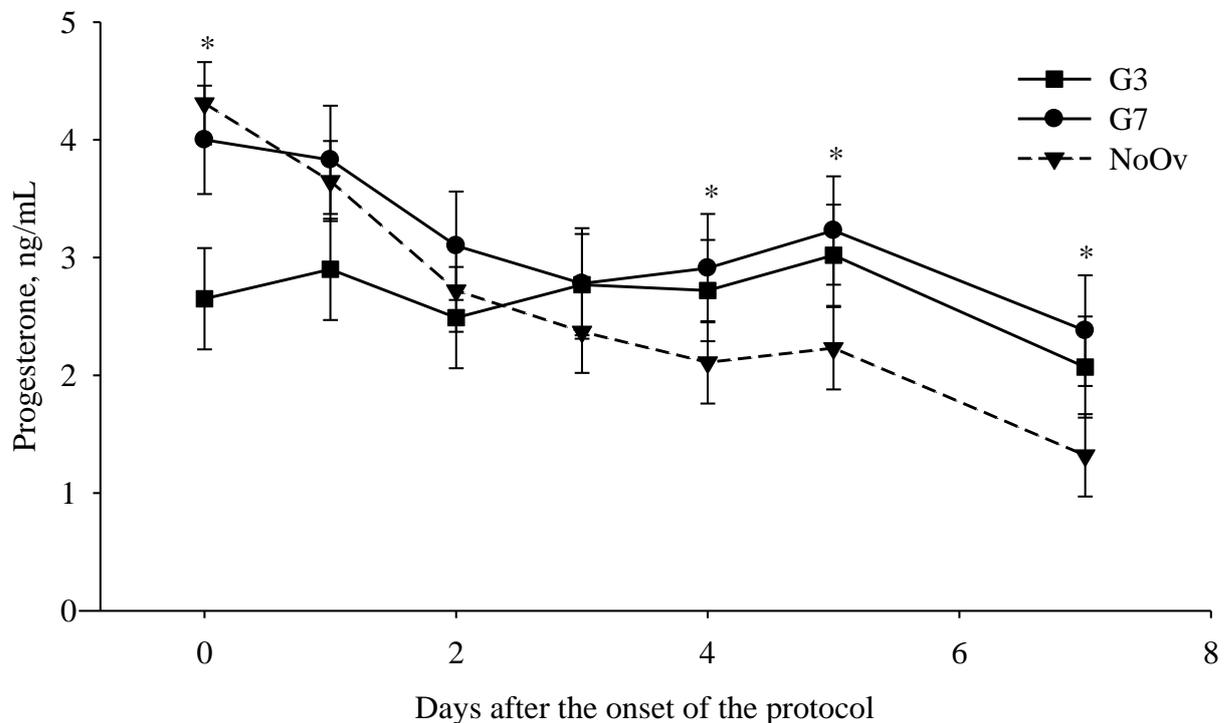


Figure 2.4 – Plasma P4 concentrations during the first 7 d after initiation of the FTAI protocol. **G3**: cows that ovulated after receiving GnRH 3 d before protocol initiation ($n = 24$); **G7**: cows that ovulated after receiving GnRH 7 d before protocol initiation ($n = 20$); **NoOv**: cows that did not ovulate to GnRH given 3 d or 7 d before protocol initiation ($n = 38$). There was a tendency for a treatment effect ($P = 0.06$), effect of day ($P < 0.001$), and interaction between treatment x day ($P = 0.01$). *Treatment effect within day ($P < 0.05$)

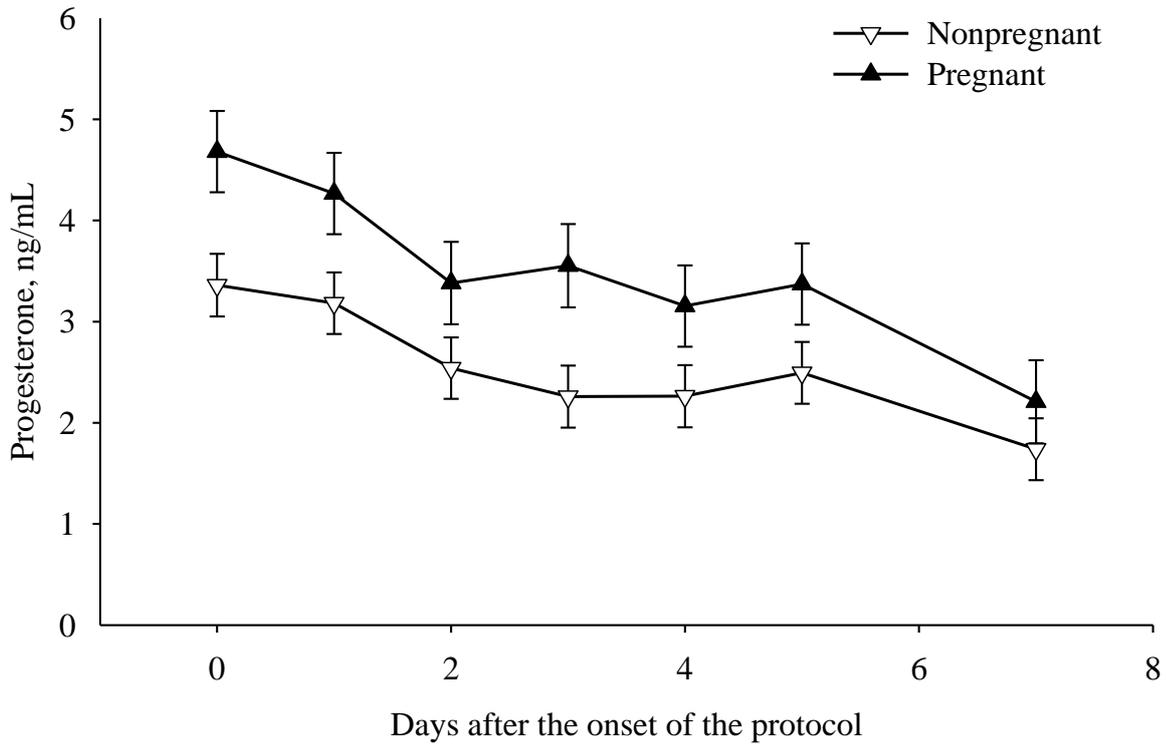


Figure 2.5 – Plasma P4 concentrations from protocol initiation (d 0) until d 7. Nonpregnant (n = 53): cows not pregnant at 59 d after fixed time AI (FTAI); Pregnant (n = 29): cows pregnant at 59 d afterFTAI . Cows received FTAI on d 10 of the protocol. From d 0 to 5 and on d 7, concentrations of P4 averaged 2.5 ± 0.23 and 3.5 ± 0.29 ng/mL for nonpregnant and pregnant cows, respectively. There were effects of pregnancy status ($P = 0.01$) and day ($P < 0.001$), but there was no interaction between pregnancy status and day

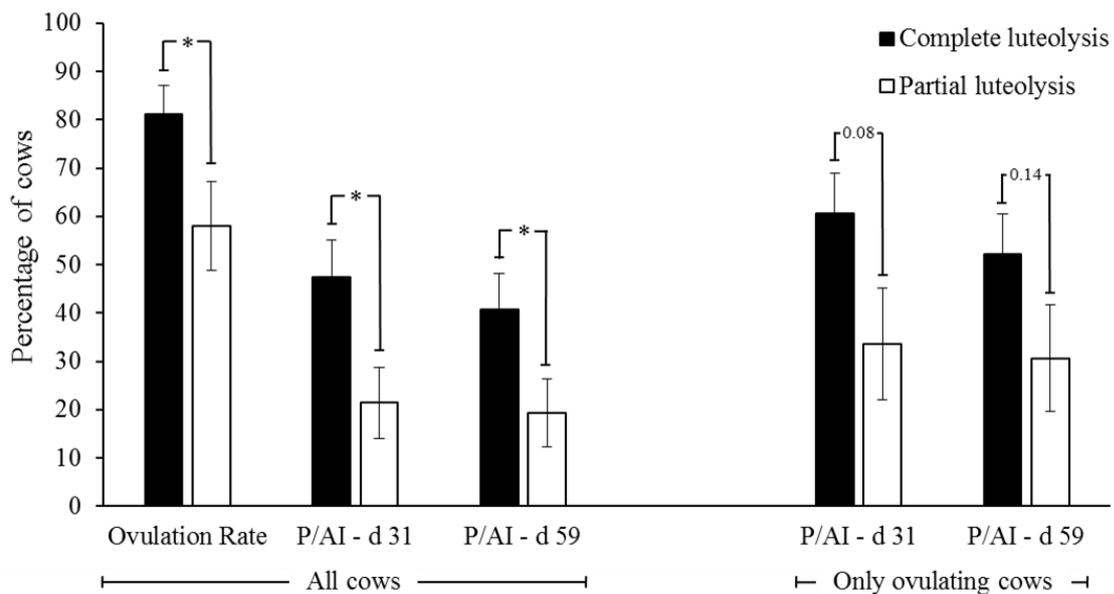


Figure 2.6 – Ovulation rate (percentage of cows that ovulated at the end of the protocol) and Pregnancy/AI (P/AI) at d 31 and d 59 after fixed time AI (FTAI) for cows that had complete luteolysis (n = 49; P4 < 0.10 ng/mL) or partial luteolysis (n = 33; P4 ≥ 0.10 and < 0.22 ng/mL) on day of FTAI. *Effect of complete vs. partial luteolysis ($P \leq 0.05$)

Finally, independent of treatment group, ovulatory follicle size had no effect on P/AI (using linear regression analysis) based on either the d 31 ($P = 0.73$) or d 59 ($P = 0.88$) pregnancy diagnosis. The P/AI was not different between cows ovulating smaller (≤ 14 mm), medium (> 14 and ≤ 17 mm) or larger (> 17 mm) follicles based on d 31 ($69.4\% \pm 12.4$ [11/17], $51.3\% \pm 12.2$; [15/29], and $58.7\% \pm 16.7$ [7/13], respectively) or d 59 ($65.6\% \pm 12.7$ [10/17], $50.0\% \pm 12.3$ [14/29] and $46.3\% \pm 17.1$ [5/13]) pregnancy diagnosis.

2.3.3 Combination of experiment 1 and 2

Results from experiment 1 and 2 were combined in order to evaluate the percentage of cows that were synchronized based on specific measures (Table 2.3). A total of 73.8% of cows (93/126) had emergence of a new follicular wave during the protocol with greater ($P = 0.01$) P/AI in cows with new wave emergence compared to cows with a persistent follicle. A total of 77.8% of cows ovulated at the end of the protocol (98/126) with no cow becoming pregnant that did not have ovulation at the end of the protocol (0/28). Therefore, overall synchronization rate, based on follicle emergence and ovulation, was 59.5% (75/126) with much greater ($P < 0.01$) P/AI in synchronized compared to non-synchronized cows (Table 2.3).

Table 2.3 – Results of the analysis of synchronization to the protocol using all cows from both experiments. Cows were classified by whether they had emergence of the new follicular wave during the protocol (d 1 to 6) and whether they had ovulation at the end of the protocol in order to determine the percentage of cows that were synchronized to the FTAI protocol and the Pregnancies/AI (P/AI) for synchronized (Yes) and non-synchronized (No) cows

	P/AI, %		<i>P</i>
	Yes	No	
Emerged new wave ¹	49.5 (46/93)	24.2 (8/33)	0.01
Ovulated to protocol ²	55.1 (54/98)	0 (0/28)	< 0.01
Cows synchronized ³	61.3 (46/75)	15.7 (8/51)	< 0.01

¹ Cows that had emergence of a new follicular wave between d 1 and d 6

² Cows that ovulated between d 9.5 and 11.5 of the protocol

³ Cows were considered synchronized when they had new wave emergence and ovulated

2.4 Discussion

Reproductive management in many parts of the world use hormonal programs that synchronize ovulation in order to allow for FTAI of all eligible cows. This research evaluated the dynamic changes in ovarian structures and reproductive hormones during modifications of an E2/P4 FTAI synchronization protocol. Our first hypothesis, that increasing the dose of EB would increase synchronization, was rejected as the higher dose of EB (3 mg vs. 2 mg) did not improve follicular synchronization but instead led to earlier regression of the CL and earlier ovulation at the end of the protocol. Although we have been unable to find other studies that have tested the specific hypothesis of dose of EB compared to synchronization, our results on increased CL regression are consistent with other research results, as discussed below. Our second hypothesis, that synchronization could be improved by initiating the E2/P4 protocol at a specific stage of a follicular wave, was proposed due to the success of presynchronization programs on fertility in GnRH-based protocols (MOREIRA et al., 2001; HERLIHY et al., 2012; WILTBANK; PURSLEY, 2014). However, we also rejected this second hypothesis, as we observed that irrespective of the stage of the follicular wave at protocol initiation, many cows did not ovulate at the end of the protocol or ovulated a persistent follicle, due to lack of emergence of a new follicular wave following EB treatment. Probably, the most interesting results from this study were found when we combined the results of both experiments to evaluate the specific physiological abnormalities that led to lack of synchronization during the protocols. Only about 60% of cows were synchronized by the E2/P4 protocol, based on emergence of a new follicular wave and ovulation at the end of the protocol. Cows that were synchronized had excellent fertility to the FTAI (~ 60%) but cows that were not synchronized had low fertility (15.7%). Although the results of these experiments are consistent with previous research, as discussed below, they provide novel physiological insights into problems and possible resolutions associated with E2/P4-based protocols for synchronizing ovulation.

Treatments with P4 and various types of E2 have been shown to result in the emergence of a new follicular wave (BO et al., 1995a, 1995b), however, consistent with the results of a previous study in lactating dairy cows (SOUZA et al., 2009), more than 25% (33/126) of dairy cows in our study did not demonstrate emergence of a new follicular wave after EB treatment. Insufficient EB does not seem to explain this problem, since increasing the dose of EB from 2.0 to 3.0 mg did not reduce the percentage of cows that ovulated a persistent follicle (29% persistent follicles after 3 mg EB; 6/21). It is unclear why some cows did not have new wave emergence after EB treatment but one possibility, that was not tested in our study, is that there

was insufficient suppression of circulating FSH/LH concentrations in response to EB and P4 treatment at protocol initiation and therefore follicular wave turnover did not occur. Lactating dairy cows have increased E2 and P4 metabolism due to elevated liver blood flow (SANGSRITAVONG et al., 2002; WILTBANK et al., 2006) and therefore the P4 implant and EB may have produced an insufficient increase in P4 and E2 to suppress gonadotropins. In addition, similar circulating P4 concentrations seem to be less inhibitory to LH pulses in lactating compared to non-lactating cows (VASCONCELOS et al., 2003).

Another important consideration is that treatment with EB, particularly the 3 mg dose of EB, was associated with regression of the CL. Increasing the EB dose from 2 mg to 3 mg clearly produced greater CL regression during the protocol as the number of cows with CL at the time of PGF treatment (d 7) decreased from about 60% with 2 mg to less than 20% after 3 mg EB treatment. We did not measure circulating P4 in this first study, however CL regression would be followed by a substantial decrease in circulating P4 and probably increased frequency of GnRH/LH pulses and possibly FSH pulses. These gonadotropin changes could underlie the rescue of some of the persistent follicles after EB treatment. A number of other studies have reported regression of the CL after treatment with EB (ARAUJO et al., 2009) or E2-17 β (FORD et al., 1975; THATCHER et al., 1986). It is unclear if 2 mg EB caused CL regression, since we did not have a control group that received no EB treatment. However, it is clear that most CL regress in response to 3 mg EB. Treatment with E2 can increase PGF production by the uterus (FORD et al., 1975; THATCHER et al., 1986). This effect appears to be mediated by binding of E2 to estrogen receptor α , subsequent upregulation of oxytocin receptor expression, binding of oxytocin to the oxytocin receptor, and subsequent production of PGF pulses due to the pulsatile pattern of oxytocin pulses (McCRACKEN; CUSTER; LAMSA, 1999; FLEMING et al., 2006; SPENCER et al., 2007). Thus, although greater doses of EB might be expected to produce greater suppression of gonadotropins, it appears this effect may be more than counteracted by the decrease in circulating P4 due to increased CL regression with a greater dose of EB. It seems likely that a better way to produce a more consistent regression of all follicles is to increase the amount of P4 simultaneously to EB treatment, such as by using high doses of injectable P4 or by including two P4 implants during the protocol, but these approaches also need to be tested.

Our observations of low fertility in cows that ovulated a persistent follicle are similar to results from numerous other studies (AHMAD et al., 1996; BLEACH; GLENCROSS; KNIGHT, 2004; CERRI et al., 2009; SANTOS et al., 2010) and are consistent with the idea

that increasing the dominance period of follicles reduces fertility in the oocyte that is ovulated from the persistent follicle. This effect may be due to premature germinal vesicle breakdown due to excessive LH stimulation of the dominant follicle (AHMAD et al., 1994; REVAH; BUTLER, 1996). Oocytes from persistent follicles are fertilized but generally undergo cessation of cellular division and embryo degeneration during the first few day of embryo development (AHMAD et al., 1994; CERRI et al., 2009). For example, ovulatory follicles that were 12 d old compared to 9 d of age had a lower percentage of freezable embryos and a greater percentage of degenerate embryos (CERRI et al., 2009). In addition, lower circulating P4 concentrations during follicle growth can result in reduced fertility of the follicle, independent of follicle age (RIVERA et al., 2011; WILTBANK et al., 2014b). This idea was supported by our data as circulating P4 concentrations were consistently greater in cows that became pregnant compared to cows that became non-pregnant, independent of treatment group (Figure 2.5). Lower fertility and increased double ovulation have consistently been observed in cows with lower circulating P4 during ovulatory follicle growth in GnRH-based synchronization protocols (LOPEZ et al., 2005; BISINOTTO et al., 2013; WILTBANK et al., 2014b). Thus, an important area of opportunity to improve E2/P4-based synchronization protocols in lactating dairy cows is to increase the percentage of cows with complete follicle suppression, emergence of a new follicular wave, and elevated circulating P4 during the protocol.

Another important hormonal problem that we observed during the protocol was that some cows had a slight elevation in circulating P4 near the time of AI (> 0.1 ng/mL). This elevation was associated with a decrease in P/AI at the d 31 and the d 59 pregnancy diagnoses. One important reason for the decrease in fertility was a decrease in ovulation at the end of the protocol in cows with small P4 elevations. Another study also reported that an elevation in circulating P4 to more than 0.1 ng/mL resulted in a reduced P/AI in lactating dairy cows treated with an E2/P4-based FTAI protocol (PEREIRA et al., 2013b). This appears to be a lower threshold for P4 than has been reported in studies using GnRH-based protocols in which the threshold for reduced fertility was between 0.3 to 0.5 ng/mL for P4 (SOUZA et al., 2007; BRUSVEEN; SOUZA; WILTBANK, 2009; BISINOTTO et al., 2010; GIORDANO et al., 2012, 2013). It seems likely that any difference in P4 threshold could relate to inhibition of an ECP-induced LH surge by low concentrations of P4, since E2 action is blocked by P4 at the hypothalamic GnRH level (ROBINSON et al., 2000; RICHTER; ROBINSON; EVANS, 2002). Thus, we observed a decrease in percentage of cows that ovulated, probably due to lack of a GnRH/LH surge in some cows with P4 above 0.1 ng/mL. In contrast, induction of ovulation by using GnRH is likely to occur, even in the presence of low concentrations of P4, however, the

threshold that inhibits gamete transport, a second mechanism that reduces fertility (DAY; POLGE, 1968; HUNTER, 1968), may be reached when circulating P4 exceeds 0.3 to 0.5 ng/mL. In our study if we evaluated only those cows that ovulated at the end of the protocol, there was still a tendency for reduced fertility (>20% reduction) in cows with a small elevation in circulating P4. Thus, other mechanisms, in addition to inhibition of ovulation, are likely to underlie part of the reduction in fertility in cows with slightly elevated P4 near AI. In these experiments, it seems likely that if we had treated cows with a second dose of PGF, as has been done in GnRH-based FTAI protocols (BRUSVEEN; SOUZA; WILTBANK, 2009; SANTOS et al., 2010; RIBEIRO et al., 2012), we could have diminished the problem of incomplete luteolysis. Future experiments need to be designed to specifically evaluate whether there is a difference in P4 threshold near AI in GnRH vs. ECP induced ovulation protocols and to differentiate the mechanisms of fertility inhibition as well as effective treatments for this problem in FTAI protocols.

A major problem that we and others (SOUZA et al., 2009; PEREIRA et al., 2013a, 2013b) have observed in the E2/P4-based FTAI protocols is that some cows do not ovulate at the end of the protocol. In our study more than 20% of cows did not ovulate at the end of the protocol and none of these cows became pregnant to the FTAI. As discussed above, over half of the cows that did not ovulate at the end of the protocol (13/23 in experiment 2), had elevated P4 that probably inhibited the ECP-induced GnRH/LH surge. In addition, some cows (9/28, both experiments) appeared to have late emergence or no emergence of a follicular wave during the protocol and therefore the dominant follicle was still small at the time of ECP treatment. A previous study in lactating Holstein dairy cows reported that the dominant follicle did not acquire ovulatory capacity until it reached a diameter of more than 10.0 mm (SARTORI et al., 2001). One focus in future studies to improve E2/P4-based FTAI protocols should be to increase ovulation at the end of the protocol.

One surprising observation was that the percentage of cows with synchronized wave emergence and ovulation of a single follicle was below 50% in both of our experiments. One reason was that about 20% of cows did not have a new follicular wave and another 20% of cows did not have ovulation at the end of the protocol, as discussed above. However, the multiple ovulation rate was also surprisingly high in these studies, particularly in experiment 1. Multiple ovulation rate is responsible for the high twinning rate in lactating dairy cows (WILTBANK et al., 2000; DEL RIO; KIRKPATRICK; FRICKE, 2006;). Although high milk production (FRICKE; WILTBANK, 1999; LOPEZ et al., 2005) and low circulating P4 prior to

AI (CERRI et al., 2011; WILTBANK et al., 2012) are clearly risk factors for increased double ovulation, there are also numerous other risk factors that could produce high double ovulation and twinning rates (KINSEL et al., 1998; DEL RIO et al., 2007).

In conclusion, although FTAI protocols using EB and P4 produce satisfactory results for lactating dairy cows, they still need improvements to provide a more consistent wave emergence synchronization, higher circulating P4 during the protocol, successful induction of complete luteolysis, as well as higher ovulation rates. Presynchronization with GnRH or increasing the dose of EB to 3 mg were not effective in providing those improvements.

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3 PROGESTERONE SUPPLEMENTATION POST OVULATION: EFFECTS ON CORPUS LUTEUM FUNCTION AND ON FERTILITY OF DAIRY COWS SUBJECTED TO AI OR ET

Abstract

In order to evaluate the effect of progesterone (P4) supplementation starting during metestrus on the formation of the corpus luteum (CL), and on the fertility of lactating dairy cows subjected to fixed time artificial insemination (FTAI) or embryo transfer (ET) were carried out three experiments. In Exp 1, 42 Holstein cows were allocated randomly to untreated (Control), or a controlled internal drug release implant containing 1.9 g of P4 from d 3 to 20 after FTAI (CIDR). The FTAI protocol consisted of placing a CIDR and administering 2 mg estradiol benzoate on d -10. On d -3, 25 mg dinoprost (PGF) and on d-2, 1 mg estradiol cypionate with CIDR removal. Blood samples were collected on d 3, 4, 7, 11, 14, 17, 20 and 21 for plasma concentration of P4 by RIA. Ultrasound scans were performed at d 4, 7, 11, 14 and 20 to calculate the CL volume. In Exp 2, 668 Holstein and crossbred cows were subjected to FTAI and allocated randomly to untreated (AI-Control) or to receive a CIDR from d 3 to 17 (AI-CIDR) after FTAI. In Exp 3, 360 Holstein cows were treated with PGF and after heat detection (d 0), they were allocated to untreated (ET-Control), or to receive a CIDR from d 4 ± 1 to 8 ± 1 (ET-CIDR-4) or a CIDR from 4 ± 1 to 18 ± 1 (ET-CIDR-14). *In vitro*-produced embryos were transferred on d 8 ± 1 . Pregnancy diagnoses were performed by ultrasound. In Exp 1 there was interaction between treatment and day in relation to plasma P4 on d 4 and d 7 due to supplementation. Independent of treatment, pregnant cows had higher plasma P4 from d 14 to d 21. Supplementation did not seem to compromise CL development. In Exp 2, there was no effect of supplementation of P4 on pregnancy per AI (P/AI) on d 32 (32.0% vs. 31.8%, for AI-Control and AI-CIDR, respectively) or pregnancy loss (15.6% vs. 17.6%, for AI-Control and AI-CIDR, respectively). In Exp 3, there was no interaction between treatment and day. However, P4 supplementation compromised pregnancy/ET (P/ET) on d 32 in both supplemented groups (39.7% vs. 21.3% vs. 15.2% for ET-Control, ET-CIDR-4, and ET-CIDR-14, respectively), with no effect on pregnancy loss. Therefore, although CIDR insertion on d 3 after FTAI did not affect CL function and increased circulating P4, it did not increase P/AI in lactating dairy cows submitted to FTAI. Moreover, P4 supplementation decreased P/ET in lactating recipient cows.

Keywords: Bovine; Fertility; Semen; Embryo; Hormone supplementation

3.1 Introduction

A decline on fertility of dairy cows has been observed on the last 40 years, in terms of increased number of artificial insemination (AI) per pregnant cow (LUCY, 2001; WASHBURN et al., 2002). This was caused by several factors, such as changes in the physiology related to higher milk production. For example, dairy cows have lesser duration and events of estrus (LOPEZ; SATTER; WILTBANK, 2004). One feature on the high-producing lactating dairy cow is high dry matter intake (HARRISON et al., 1990). Dry matter intake is negatively

correlated with plasma concentration of progesterone (P4) and estradiol (E2) (SANGSRITAVONG et al., 2002), due to increased liver blood flow (PARR et al., 1993; SANGSRITAVONG et al., 2002). Nevertheless, to improve reproductive efficiency in dairy cattle, management programs have been adopted, such as fixed time AI (FTAI), that has been used wide world (BISINOTTO; RIBEIRO; SANTOS, 2014). Moreover, and in spite of the negative effects of the high milk production and heat stress on the oocyte quality (SARTORI; BASTOS; WILTBANK, 2010), some studies have shown increased fertility when embryo transfer (ET) in dairy cattle was used in substitution to AI (DEMETRIO et al., 2007; STEWART et al., 2011).

In lactating dairy cows, high circulating P4 during follicle development (BISINOTTO et al., 2013; SOUZA et al., 2008) and after AI (PARR et al., 2012) has been shown to be associated with increased fertility. Supplemental P4 has been used before (BISINOTTO et al., 2013; SOUZA et al., 2008) and after AI (WILTBANK et al., 1956; JOHNSON; ROSS; FOURT, 1958; LARSON; BUTLER; CURRIE, 2007; MONTEIRO et al., 2014) either by the use of intravaginal P4 implants (WILTBANK et al., 1956; JOHNSON et al., 1958; LARSON et al., 2007; BISINOTTO et al., 2013; MONTEIRO et al., 2014) or by induction of accessory corpus luteum (CL) (SANTOS et al., 2001; NASCIMENTO et al., 2013). This strategy has been associated with increased pregnancy/AI (P/AI). Additionally, cows subjected to P4 supplementation 4 d before *in vitro*-produced (IVP) embryo transfer had bigger conceptuses on d 14 (CLEMENTE et al., 2009; O'HARA et al., 2014), and higher interferon- τ (IFN- τ) concentration in the uterine lumen (MANN; FRAY; LAMMING, 2006). Nevertheless, supplementation of P4 after AI during metestrus or early diestrus has been associated with slightly shorter estrous cycles (LAWSON; CAHILL, 1983) and greater proportion of cows with P4 concentration below 1.0 ng/mL on d 19 of the estrous cycle (MONTEIRO et al., 2014).

The objectives of the present study were to investigate the effects of P4 supplementation, post ovulation, on CL function, on P/AI and on pregnancy/ET (P/ET). Therefore, three experiments were conducted to test the hypotheses that P4 supplementation 3 d after FTAI do not interfere with CL development and function, and increases fertility in dairy cows. In addition, we hypothesized that P4 supplementation 4 d before ET improves P/ET.

3.2 Materials and Methods

All procedures with cows were approved by the Ethics Committee for Animal Use of the University of São Paulo/ESALQ.

3.2.1 Experiment 1

Cows and Housing

This study was conducted on a dairy farm in southeastern region of Brazil using 64 lactating Holstein cows. Primiparous (n = 27) and multiparous (n = 37) cows (35.3 ± 10.60 kg/d of milk, 170.6 ± 180.97 DIM, and body condition score [BCS] of 2.71 ± 0.30 [mean \pm standard deviation]) were enrolled in this experiment. Cows were housed in free-stall barns, milked three times daily, and fed TMR typical of high producing herds. This experiment was conducted during the end of winter and beginning of spring (August and September).

Experimental Design, Ultrasonography Evaluation and Treatments

All cows were synchronized and received FTAI consisted of 2.0 mg, im, of estradiol benzoate (**EB**, Gonadiol, MSD Saúde Animal, São Paulo, Brazil) associated with a controlled internal drug release (**CIDR**, Zoetis, São Paulo, Brazil) insert containing 1.9 g of P4 (d -10). Seven d later (d -3) cows received 25 mg, im, of dinoprost tromethamine (**PGF**, Lutalyse, Zoetis, São Paulo, Brazil), and a day after (d -2), cows received 1.0 mg, im, of estradiol cypionate (**ECP**, Zoetis, São Paulo, Brazil) and the CIDR was removed. All cows were inseminated on d 0 (Figure 3.1).

Cows had their ovaries examined by transrectal ultrasound (DP-2200, Mindray, Huntingdon, United Kingdom) with a 7.5 MHz linear-array transducer, performed every 24 h, beginning on d -3 until ovulation or d 2 to confirm ovulation. Only cows that ovulated between d -1 and d 1 were included in the study. On d 3, ovulated cows were randomly assigned to two treatments: **Control** (n = 21), cows were not supplemented with P4; or **CIDR** (n = 21), cows received a CIDR on d 3 after FTAI which was retained until d 20 after FTAI (Figure 3.1). In these 42 ovulated cows, ovarian structures on d 4, d 7, d 11, d 14 and d 20 were scanned and mapped. Ultrasound measurements of CL were used to calculate average diameters (average of length [L] and width [W]) and volume (V). Volume was calculated with the formula $V = 4/3 \times \pi \times R^3$ using radius (R) calculated by the formula $R = (L/2 + W/2)/2$. For a CL with a fluid-filled cavity, the cavity volume was calculated and subtracted from the total volume of the CL (SARTORI et al., 2004).

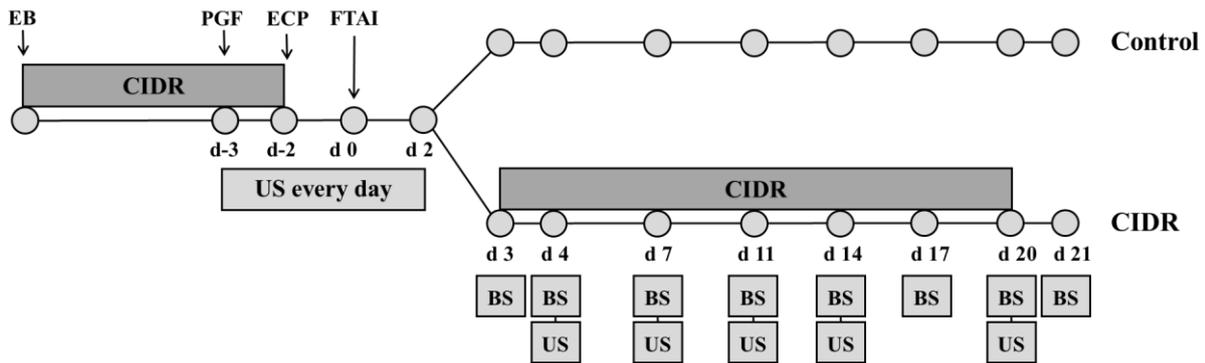


Figure 3.1 – Diagram of activities for experiment 1. Cows were submitted to fixed time artificial insemination (FTAI) protocol, and from d -3 until d 2 cows were scanned by ultrasound (US) for confirmation of ovulation. Control (n = 21) = cows did not receive controlled internal drug-release (CIDR) implant containing 1.9 g of progesterone (P4) after FTAI; or CIDR (n = 21) = cows received a CIDR from d 3 to 20 after FTAI. BS – Blood sample

Blood Sampling and Analysis of Progesterone in Plasma

In all 42 ovulated cows, blood samples were collected by venipuncture of the median caudal vein or artery into evacuated tubes containing Sodium Heparin (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) after FTAI on d 3, immediately before administration of treatment in CIDR group, d 4, d 7, d 11, d 14, d 17, d 20, immediately before CIDR removal, and on d 21, for later analysis of plasma P4 concentration. Immediately after collection, samples were placed in ice and kept refrigerated until transport to the laboratory within a period of 5 h. Samples were centrifuged (1,700 x g) for 15 min, and plasma were harvested and stored at -20 °C until assayed for P4 using a solid-phase, no-extraction radioimmunoassay (Coat-a-Count Progesterone, Diagnostic Products Corp., Los Angeles, CA). Only a single assay was performed and the intra-assay CV was 4.2%.

Pregnancy Diagnosis

Thirty-one d after FTAI, pregnancy diagnoses were performed by ultrasound (DP-2200, Mindray) by transrectal exam. Cows with embryonic vesicle were eligible to be pregnant. All pregnant cows were reconfirmed on d 59 after FTAI. The difference between pregnant cows on d 31 and pregnant on d 59 was used to calculate pregnancy loss.

3.2.2 Experiment 2

Cows and Housing

Six-hundred and sixty eight cows (23.6 ± 9.87 kg/d of milk, 164.8 ± 123.19 DIM, and BCS of 2.74 ± 0.26 [mean \pm SD]), 330 Holsteins (160 primiparous and 170 multiparous) and 338 crossbred Holstein x Gyr (138 primiparous and 200 multiparous), milked three times a day, from three dairy farms in the southeastern region of Brazil were assigned to the study from May to December. Farm 1 (n = 255) was a grazing system and cows had access to pastures of Mombaça guinea grass (*Panicum maximum* Jacq. cv. Mombaça) and were supplemented with corn silage, chopped sugar cane, and grain mix containing ground corn, soybean meal, citrus pulp, whole cottonseed, minerals and vitamins. Farm 2 (n = 179) was also a grazing system and cows had access to pastures of Tifton 85 Bermuda grass (*Cynodon* spp.) and were supplemented with a mixture of corn silage and a grain mix containing finely ground corn, soybean meal, citrus pulp, whole cottonseed, minerals and vitamins. Farm 3 (n = 234) was a confinement system with free-stall barns and cows were fed a TMR containing corn silage, finely ground corn, soybean meal, whole cottonseed, minerals and vitamins.

Experimental Design and Treatments

On all farms, cows were synchronized and received FTAI consisted of 2.0 mg of EB, im, associated to a CIDR (d -10). Seven d later (d -3) cows received PGF, im, and 1.5 d after (d -1.5), cows received 1.0 mg of EB, im, associated to a second dose of PGF, im, and CIDR was removed. All cows were inseminated on d 0 (Figure 3.2).

On d 3 after FTAI, cows were randomly assigned to two treatments: **AI-Control** (n = 359), cows were not supplemented with P4; or **AI-CIDR** (n = 309), cows received a single CIDR until d 17 (Figure 3.2).

Pregnancy Diagnosis

In all farms, 32 d after FTAI, pregnancy diagnoses were performed by transrectal ultrasound (DP-2200, Mindray). Cows with embryonic vesicle were eligible to be pregnant. All pregnant cows were reconfirmed at 60 d after FTAI on all three farms by transrectal palpation of the uterus.

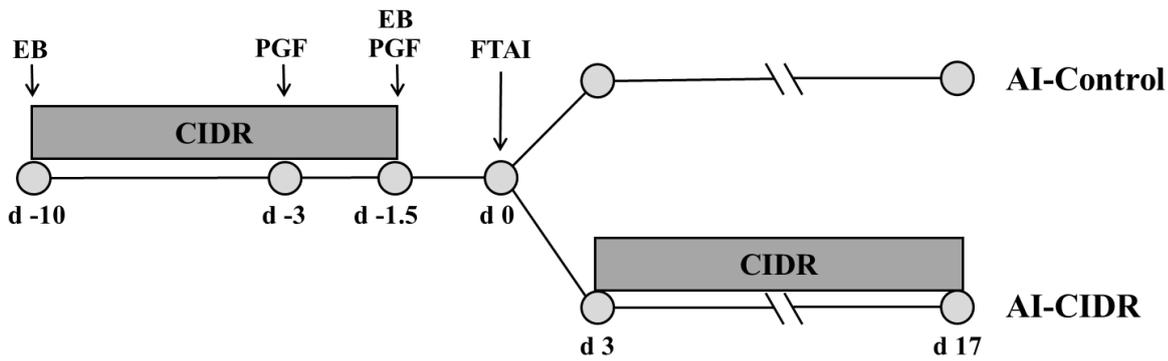


Figure 3.2 – Diagram of activities for experiment 2. On d -10 cows received estradiol benzoate (EB) and controlled internal drug-release (CIDR) implant containing 1.9 g of progesterone. On d -3 and d -1.5 cows received prostaglandin $F_{2\alpha}$ (PGF), and at the last PGF EB was administered. On d 0 fixed time AI (FTAI) was performed in all cows. On d 3, cows were randomized in AI-Control (n = 359), cows did not receive CIDR after FTAI; or AI-CIDR (n = 309), cows received a CIDR from d 3 to 17 after FTAI

3.2.3 Experiment 3

Cows and Housing

Three-hundred and sixty lactating Holstein cows (28.9 ± 10.11 kg/d of milk and 206.8 ± 141.19 DIM [mean \pm SD]), primiparous (n = 184) and multiparous (n = 176), milked three times a day, from a dairy farm in the southeastern region of Brazil were assigned in this study from June to December. Cows were housed in free-stall barns and fed a TMR containing corn silage, finely ground corn, soybean meal, whole cottonseed, minerals and vitamins.

Donors and Ovum Pick-Up

One hundred eighty five lactating dairy cows were subjected to ovum pick-up (OPU) sessions in the morning of d 0 of the recipients' protocol and had their perineal area cleaned with water and 70% ethanol. Epidural anesthesia was performed with 5 mL of 2% lidocaine hydrochloride (Xylestesin, Cristália, Itapira, Brazil) to facilitate the handling of the ovaries through the rectum. All follicle greater than 2.0 mm were aspirated by ultrasound (SSD 500, Hitachi Aloka Medical, Ltda, Wallingford, US) equipped with a 7.5-MHz convex array transducer and coupled to a vaginal aspirate guide with a stainless steel needle guide connected to a vacuum system (BV 003d, Watanabe Tecnologia Aplicada, Cravinhos, Brazil). Follicular puncture was performed using to circuit, 1.1 mm, i.d. x 1.20 m length, connected to a disposable (18-ga) hypodermic needle by vacuum pressure of 146 mmHg. Collection medium was TCM 199 (Gibco Life Technologies, Grand Island, NY, US) supplemented with 25 mM HEPES

(Sigma Cat. No. H0763), 5% fetal calf serum, 50 µL/mL gentamycin sulfate (Sigma Cat. No. G 1264), and 10,000 IU/L sodium heparin (Sigma Cat. No. H3149). Cumulus oocyte complexes (COC) were separated and, classified according to the number of cumulus cell layers and cytoplasm homogeneity: Grade 1, more than three layers and homogeneous cytoplasm; Grade 2, at least one layer of cumulus cells and homogeneous cytoplasm; Grade 3, denuded, partly covered with cumulus cells, or without cumulus cells; and Grade 4, atretic, with dark cumulus oophorus and signs of cytoplasm degeneration. Only Grade 1 and 2 COC were used for IVP.

In Vitro Embryo Production

Before *in vitro* maturation, cumulus oocyte complexes were washed three times in HEPES-buffered TCM-199, supplemented with 10% fetal calf serum and 50 µg/mL gentamycin, and once in maturation medium, composed of bicarbonate-buffered TCM-199 (Gibco Life Technologies) supplemented with 10% fetal calf serum, 50 µg/mL LH (APL, Ayrest, Rouses Point, NY), 5 µg/mL FSH (Folltropin-V, Bioniche Animal Health, Canada), 0.1 µg/mL estradiol (Estradiol-β, Cat. No. E8875), 2.2 µg pyruvate (Sigma Cat. No. P4562) and 50 µg/mL gentamycin sulfate. The cumulus oocyte complexes were cultured for 24 h in 100 µL of maturation medium under mineral oil (D'Altamore, Santo Amaro, São Paulo, Brazil) at 39 °C and 5% CO₂ in air.

Female sex-sorted semen were thawed for 30 s at 35 °C and deposited on a 90% to 45% Percoll gradient prepared with sperm wash medium (modified Tyrode medium) and centrifuged at 320 x g for 30 min to separate the motile sperm. Sperm pellet was evaluated for motility and concentration. The final concentration used was 1.0 x 10⁶ live sperm/mL, and each fertilization droplet received 5 µL (5,000 sperm cells). Cumulus oocyte complexes and sperm were incubated at 38.5 °C in an atmosphere of 5% CO₂ for 18 to 20 h.

After 18 h of the insemination, presumptive zygotes had their cumulus cells removed by mechanical pipetting and were cultured to 100 µL drops of embryo culture medium (Synthetic Oviduct Fluid containing 8 mg/mL bovine albumin serum and 1 mM glutamine) under 38.5 °C in an atmosphere of 5% CO₂ for 18 to 20 h. Until transfer, the embryos were kept under these conditions.

Experimental Design, Recipient's Protocol and Treatments

Recipient cows were synchronized with a single dose of PGF, im, 11 d before ET, and only cows that showed estrus signs between 3 and 5 d after PGF (d 0 to d -2) received ET.

Therefore, these cows were on d 7, d 8 or d 9 of the estrous cycle. Four d before ET, cows were randomly assigned to one of three treatments: **ET-Control** (n = 132), cows were not supplemented with P4; **ET-CIDR-4** (n = 119), cows received a CIDR 4 days before ET and CIDR was removed at the time of ET, 4 d of P4 supplementation; or **ET-CIDR-14** (n = 109) cows received a single CIDR 4 d before ET until 10 d after ET, 14 d of P4 supplementation (Figure 3.3). Prior to ET, recipient cows received an epidural anesthesia with 5 mL of 2% lidocaine hydrochloride and fresh embryos were transferred nonsurgically into the uterine horn ipsilateral to the CL.

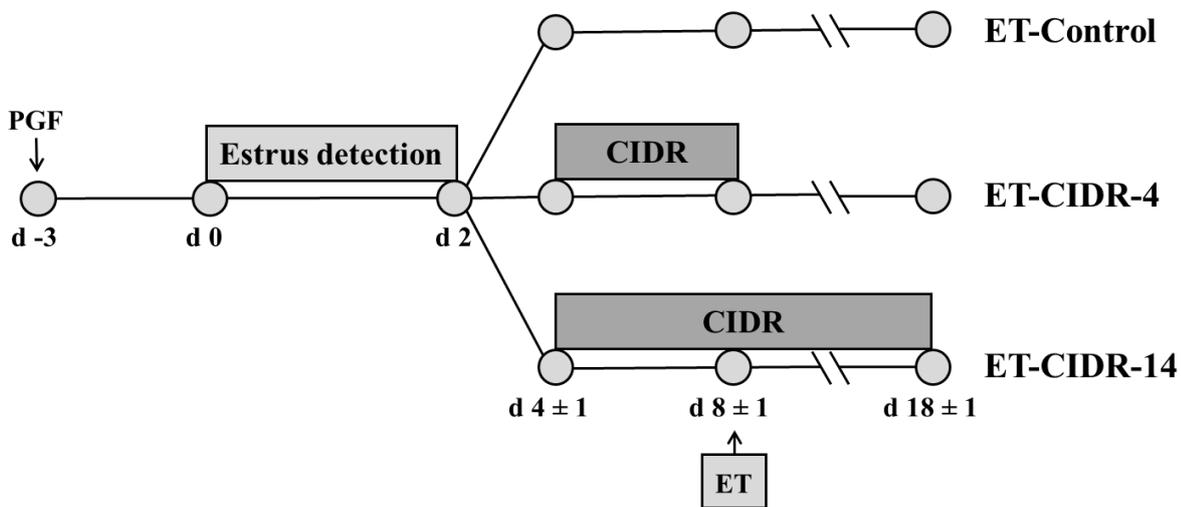


Figure 3.3 – Diagram of activities for experiment 3. On d -3 recipient cows received prostaglandin $F_{2\alpha}$ (PGF), and estrus detection was performed. Cows that showed estrus on d 0 to d 2 were randomly assigned on the study into three treatments, ET-Control (n = 132), cows did not receive a controlled internal drug-release (CIDR) implant containing 1.9 g of progesterone (P4); ET-CIDR-4 (n = 119), cows received a CIDR from d 4 ± 1 until the day of the embryo transfer (ET); or ET-CIDR-14 (n = 109), cows received a CIDR from d 4 ± 1 until 18 ± 1 . All recipient cows received embryo on d 8 ± 1

Pregnancy Diagnosis

After 25 d of ET, 32 d of possible pregnancy, pregnancy diagnosis was performed via transrectal by ultrasound (DP-2200, Mindray). Cows with embryonic vesicles were eligible to be pregnant. All pregnant cows were reconfirmed at 81 d after ET, 88 d of pregnancy, by transrectal palpation of the uterus.

3.2.4 Statistical Analysis

In experiment 1, continuous data with repeated measures over time, like circulating P4 and CL volume, were analyzed by logistic regression using the GLIMMIX procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC) with models fitting a Gaussian distributions. Data were tested for normality of residuals by Shapiro-Wilk test. The model included the fixed effects of treatment, day of measurement, breed, parity, pregnancy status on d 31, BCS categorized (above or equal to 2.75 and below), DIM categorized as above or below the mean value, milk yield categorized during the week of d 0 as above or below the mean value, interaction between treatment and day, treatment and pregnancy status, day and pregnancy status, and treatment by day by pregnancy status. When the F-test for an interaction was significant, means were then partitioned using the SLICE command in SAS.

Categorical data of experiments 2 and 3 were analyzed using the GLIMMIX procedure of SAS fitting a binary distribution. For experiment 2, the model included the fixed effects of treatment, parity, breed categorized (Holstein or crossbred Holstein x Gyr), farm, sire, DIM categorized as above or below the mean value, milk yield categorized during the week of d 0 as above or below the mean value, and interaction between parity and treatment, as well as the random effects of cows.

For experiment 3, the model included the fixed effects of treatment, parity, DIM categorized as above or below the mean value, milk yield categorized during the week of d 0 as above or below the mean value, day of estrous cycle of recipients, d 7, d 8, or d 9, and interaction between treatment and day of estrous cycles, as well as the random effects of cows.

Only variables with $P < 0.20$ were kept in the final model, unless the variable was essential, for example treatment, day, day of estrous cycle (experiment 3), interaction between treatment and day (experiment 1). The Kenward-Roger method was used to calculate the denominator degrees of freedom to approximate the F test in the mixed models. Differences with $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ were considered tendencies. Results are shown as least squares means and standard error.

3.3 Results

3.3.1 Experiment 1

Ovulation rate to FTAI protocol was 65.6% (42/64). From 42 cows used in the experiment, the proportion of ovulation on d -1, d 0 and d 1 was 9.5% (4/42), 73.8% (31/42) and 16.7% (7/42), respectively.

Mean concentrations of P4 was similar between the two treatment groups throughout the experimental period ($P = 0.14$), but there were effects of day ($P < 0.001$) and interaction among treatment \times day ($P < 0.001$; Figure 4A). Supplemented cows had concentration of P4 on d 4 ($P < 0.001$) and d 7 ($P < 0.01$) after FTAI higher than cows that did not receive supplementation. For the remainder days, the concentrations of P4 did not differ between treatments. As anticipated, concentrations of P4 did not differ between treatments on d 3. Inclusion of a CIDR on d 3 after FTAI increased concentrations of P4 by approximately 1.1 ng/mL on d 4 (1.0 vs. 2.1 ng/mL, for Control and CIDR, respectively), 1 d after treatment.

Independent of treatment, pregnant cows on d 31 after FTAI had mean concentration of P4 between d 3 and d 21 higher (4.5 ± 0.23 vs. 3.2 ± 0.27 , $P < 0.001$) than nonpregnant cows. Moreover, there was an interaction between pregnancy status and day ($P < 0.01$). Pregnant cows on d 31 had mean concentration of P4 on d 14, d 20 and d 21 after FTAI higher ($P < 0.02$) than nonpregnant cows. On d 17 pregnant cows had a tendency ($P = 0.07$) to have higher circulating P4 than nonpregnant cows (Figure 3.4A).

There was not effect of treatment ($P = 0.66$) and interaction between treatment and day ($P = 0.31$), but independent of treatment, there was effect of day on the CL volume ($P < 0.001$; Figure 4B). The CL on d 4 was smaller than on the other days. No effect ($P = 0.19$) was observed of pregnancy status on CL volume (Figure 3.4B).

Considering only ovulated cows, the P/AI on d 31 ($47.6\% \pm 11.21$ [10/21] vs. $66.7\% \pm 10.54$ [14/21] for Control and CIDR, respectively; $P = 0.23$) and d 59 ($38.1\% \pm 10.88$ [8/21] vs. $61.9\% \pm 10.88$ [13/21] for Control and CIDR, respectively; $P = 0.14$), and pregnancy loss ($19.7\% \pm 13.31$ [2/10] vs. $7.0\% \pm 6.98$ [1/14] for Control and CIDR, respectively; $P = 0.40$) did not differ between treatments.

3.3.2 Experiment 2

The P/AI for AI-Control and AI-CIDR did not differ on d 32 ($32.0\% \pm 2.55$ [115/359] vs. $31.8\% \pm 2.75$ [98/309], respectively), neither on d 60 ($25.9\% \pm 2.83$ [95/359] vs. 25.0 ± 2.96 [79/309], respectively). Independent of treatment, primiparous cows had better fertility than multiparous on d 32 ($P = 0.03$) and a tendency ($P = 0.06$) to be better on d 60. An interaction ($P = 0.04$) between treatment and parity was observed for P/AI on d 32. Primiparous cows supplemented with P4 had higher P/AI than supplemented multiparous cows. No difference was observed between not supplemented primiparous and multiparous cows when compared with supplemented primiparous or multiparous cows. There was no effect of treatment or interaction between treatment and parity on P/AI at d 60 (Table 1).

There was also no effect of treatment ($15.6\% \pm 3.69$ [20/115] vs. $17.6\% \pm 4.29$ [19/98] for AI-Control and AI-CIDR, respectively) on pregnancy loss between d 32 and d 60, but there was a tendency ($P = 0.08$) for interaction between treatment and parity. Supplemented primiparous cows tended to present higher pregnancy loss than supplemented multiparous cows (Table 1).

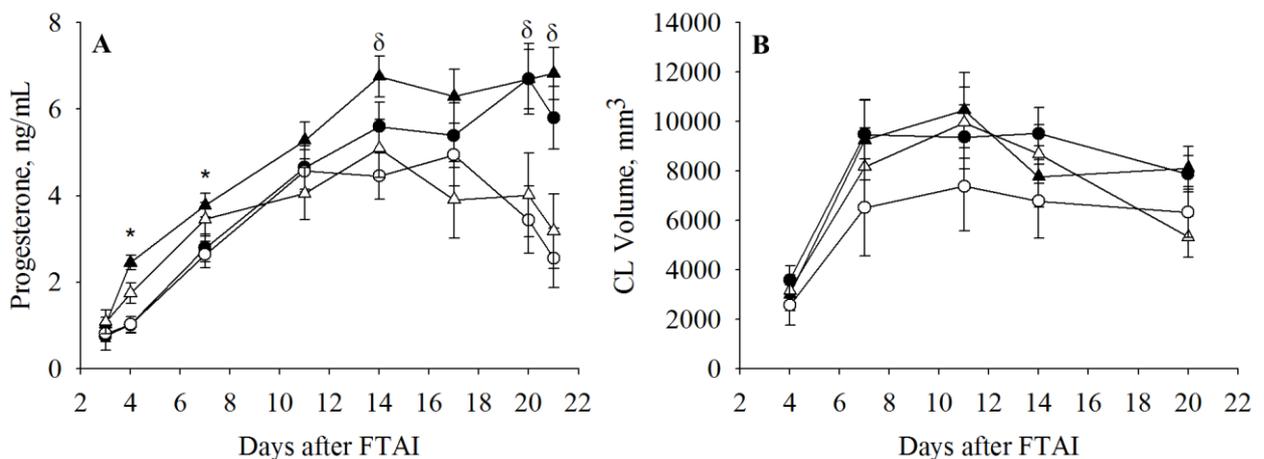


Figure 3.4 – Concentration of progesterone (P4) in plasma (A) and corpus luteum (CL) volume (B) according to the day after AI and pregnancy status on experiment 1. (●) cows did not receive a controlled internal drug-release (CIDR) implant containing 1.9 g of P4 and were pregnant on d 31 after FTAI ($n = 10$); (○) cows did not receive a CIDR and were not pregnant on d 31 after FTAI ($n = 11$); (▲) cows received a CIDR from d 3 to 20 after FTAI and were pregnant on d 31 after FTAI ($n = 14$); (△) cows received a CIDR from d 3 to 20 after FTAI and were not pregnant on d 31 after FTAI ($n = 7$). Only ovulated cows were enrolled on the experiment. There was no effect of treatment ($P = 0.14$), but there was effect of day ($P < 0.001$) and interaction between treatment and day ($P < 0.01$) on the concentration of P4. There was no effect of treatment ($P = 0.66$) and no interaction between treatment and day ($P = 0.31$), but there was effect of day ($P < 0.001$). *Within a day, effect of treatment on the concentration of P4. δ Within a day, effect of pregnancy status on the concentration of P4

3.3.3 Experiment 3

Both supplemented groups, ET-CIDR-4 and ET-CIDR-14, had lower proportion of pregnant cows on d 32 ($P < 0.001$) and d 88 ($P = 0.001$) than ET-Control. However, pregnancy loss between d 32 and d 88 did not differ among treatments (Table 3). There was no interaction between treatment and parity on P/ET on d 32 and d 88, neither on pregnancy loss.

There was no effect ($P > 0.10$) of ET day in relation to day of the estrous cycle of recipients on P/ET on d 32 ($27.4\% \pm 4.18$ [$n = 128$], $24.8\% \pm 38.30$ [$n = 143$] and $20.4\% \pm 4.40$ [$n = 89$], for d 7, d 8 and d 9, respectively) and on d 88 ($19.0\% \pm 3.67$, $14.6\% \pm 3.10$ and 14.1 ± 3.78 , for d 7, d 8 and d 9, respectively). Pregnancy loss between d 32 and d 88 of pregnancy was similar among the cows that received embryo on d 7, d 8, and d 9 of the estrous cycle ($28.5\% \pm 8.16$, $38.4\% \pm 8.97$ and $29.2\% \pm 11.12$, respectively). Additionally, it was not observed interaction between treatment and day of the estrous cycle of recipient cows.

3.4 Discussion

This study evaluated the effect of supplementation of P4 3 d after FTAI or 4 d before ET in lactating dairy cattle. Our first hypothesis, that supplementation with P4 3 d after FTAI would not change CL development and function, was accepted since the insertion of a CIDR 3 d after FTAI increased plasma P4 concentration on d 4 and d 7. Additionally up to CIDR removal on d 21, the concentration of P4 between treatments was similar throughout the period. Our second hypothesis, that supplementation of P4 3 d after FTAI would increase P/AI of dairy cows was rejected, since the P/AI between treatments was similar (32.0% vs. 31.8% , on d 32, for AI-Control and AI-CIDR, respectively), but there was interaction between treatment and parity, as discussed below. Our third hypothesis, that supplementation of P4 4 d (± 1) before ET would increase P/ET was proposed due to other studies that reported increase of embryo area in recipients supplemented with P4 before receiving the embryo (O'HARA et al., 2014) and with higher concentration of IFN in the uterus (MANN; FRAY; LAMMING, 2006). However, we also rejected this hypothesis, since P/ET was lower in P4-supplemented recipients, regardless of time of supplementation (4 or 14 d) in relation to the unsupplemented group.

Table 3.1 – Effect of the supplemental progesterone (P4) after fixed time artificial insemination (FTAI) on fertility responses in lactating dairy cows according to parity

	Primiparous		Multiparous		<i>P</i> -value ³		
	AI-Control ¹	AI-CIDR ²	AI-Control ¹	AI-CIDR ²	TRT	Parity	TRT x Parity
	Adjusted proportions ± SE (n/n)						
Pregnant/AI, %							
d 32	32.3 ± 3.88 ^{ab} (49/152)	40.2 ± 4.16 ^a (58/146)	31.7 ± 3.31 ^{ab} (66/207)	24.4 ± 3.42 ^b (40/163)	0.95	0.03	0.04
d 60	27.4 ± 4.13 (43/152)	30.8 ± 4.36 (44/146)	24.5 ± 3.44 (52/207)	19.9 ± 3.49 (35/163)	0.80	0.06	0.26
Pregnancy loss⁴, %	12.1 ± 4.88 (6/49)	24.3 ± 5.99 (14/58)	19.8 ± 5.09 (14/66)	12.4 ± 5.37 (5/40)	0.72	0.77	0.08

¹ AI-Control = cows received no supplemental P4

² AI-CIDR = cows received a controlled internal drug-release (CIDR) implant containing 1.9 g of P4 from d 3 to 17 after FTAI

³ TRT = effect of supplemental P4; Parity = effect of parity, primiparous or multiparous cows; TRT x Parity = interaction between supplemental P4 and parity

⁴ Pregnancy loss between d 32 and 60 of gestation

Table 3.2 – Effect of the supplemental progesterone (P4) in embryo recipient dairy cows 4 d before embryo transfer (ET) on fertility

	ET-Control ¹	ET-CIDR-4 ²	ET-CIDR-14 ³	P-value
	Adjusted proportions ± SE (n/n)			
Pregnant/ET, %				
d 32	39.7 ± 4.47 ^a (53/132)	21.3 ± 3.89 ^b (26/119)	15.2 ± 3.51 ^b (17/109)	< 0.001
d 88	27.9 ± 4.15 ^a (37/132)	11.5 ± 3.00 ^b (14/119)	11.7 ± 3.18 ^b (13/109)	0.001
Pregnancy loss,⁴ %	29.5 ± 6.69 (16/53)	45.7 ± 10.53 (12/26)	22.6 ± 10.59 (4/17)	0.27

¹Control = cows received no supplemental P4

²ET-CIDR-4 = cows received a controlled internal drug-release (CIDR) implant containing P4 4 d before receiving an embryo. CIDR was removed at ET

³ET-CIDR-14 = cows received a CIDR containing 1.9 g P4 4 d before receiving an embryo. CIDR was removed on d 10 after ET

⁴Pregnancy loss between d 32 and 88 of gestation

In experiment 1, there was a transient increase in plasma P4 concentration on d 4 and d 7 in P4-supplemented cows. The effect of P4 supplementation on the conceptus development seems to be effective starting at the third d of the estrous cycle (O'HARA et al., 2014). Although we were able to detect a difference in circulating P4 after CIDR insertion, after d 7, the concentration of P4 was similar between treatments. This probably happened because of the continued increase in P4 production by the CL throughout the cycle, and that the P4 device had decreased its P4 release gradually due to the time of use (CERRI et al., 2009). The similar concentration of P4 between treatments on d 21, a day after CIDR removal, supports the idea that P4 supplementation did not compromise CL function. Despite knowing that there are LH receptors in the CL (FITZ et al., 1982), and that increases circulating P4 decreases LH pulse frequency (ROBINSON et al., 2000), the supplementation of P4 3 d after AI did not seem to have impaired CL formation. Additionally, studies with LH receptors on CL showed that small luteal cells have approximately 11 times more LH receptors than large luteal cells. Probably because of this, under *in vitro* conditions, small luteal cells, when challenged with LH, increased P4 concentration in 1240%, whereas large luteal increased only 20% (FITZ et al., 1982). However, depending on the day of the estrous cycle, the CL of ruminants have 3.0 to 4.6 times more small luteal cells than large luteal cells (RODGERS; OSHEA; BRUCE, 1984; FARIN et al., 1986). Small luteal cells are responsible for 17.5% of CL volume, while large luteal cells

represent 25.4% (RODGERS; OSHEA; BRUCE, 1984). Nevertheless, small luteal cells produce approximately 15 times less P4 than large luteal cells (FITZ et al., 1982).

Although other studies (WILTBANK et al., 1956; JOHNSON et al., 1958; SANTOS et al., 2001; LARSON et al., 2007) have shown benefits of supplemental P4 after AI, in the present study, no effect was observed on the fertility of dairy cows. In fact, an interaction and a tendency for interaction between supplementation of P4 and parity was observed on the P/AI at d 32 and pregnancy loss, respectively. Cows supplemented with P4 after ovulation had an improvement on embryo development (CLEMENTE et al., 2009; O'HARA et al., 2014), and although nonpregnant cows presented a shortened estrous cycle than not supplemented cows, there was a positive effect of P4 supplementation at d 4 after FTAI on P/AI (MONTEIRO et al., 2014). Additionally, when pregnant primiparous cows were compared to pregnant multiparous cows, they had greater expression of stimulated IFN- τ genes on d 19 than multiparous (RIBEIRO et al., 2014). This is indicative that the IFN- τ secreted by the elongating conceptus may inhibit the luteolytic mechanism (SPENCER et al., 1995) in primiparous cows because it happens before - probably some hours or a day before - than in multiparous cows. Nevertheless, due to the advancement of luteolytic mechanism in supplemented cows (LAWSON; CAHILL, 1983; MONTEIRO et al., 2014), probably, in multiparous cows, luteolysis had started before the time of maternal recognition of pregnancy. On the other hand, in primiparous cows, the embryo had time to inhibit luteolysis. However, a tendency observed on the unexpected increased pregnancy loss in P4-supplemented primiparous cows resulted in similar P/AI on d 60 among groups.

Although IVP embryo is a model widely used to study the effect of P4 supplementation on embryo development (MANN et al., 2006; LONERGAN et al., 2007; CARTER et al., 2008; CLEMENTE et al., 2009), in our study supplementation of P4 for lactating recipient dairy cows had negative effect on fertility. Recipients supplemented with P4 had 50% or less P/ET than the control group. The exact mechanism related to P4 supplementation and decreased fertility in recipients is still unknown, but we believe that this may be associated with a delayed expression of IFN- τ of *in vitro* as compared with *in vivo*-produced embryos (BERTOLINI et al., 2002). Similarly to what happened with FTAI multiparous cows, embryo recipient dairy cows may have had luteolysis before enough IFN- τ secretion by the embryo. An alternative hypothesis for the decreased fertility in P4-supplemented embryo recipients is vaginitis caused due to the presence of the intravaginal P4 device at the time of embryo transfer, potentially resulting in uterine contamination.

In conclusion, although supplementation of P4 to lactating dairy cows with intravaginal inserts post ovulation increased the concentration of P4 on d 4 and d 7 without compromising CL volume and circulating P4, it did not improve P/AI in cows subjected to FTAI. Moreover, P4 supplementation to embryo recipient lactating dairy cows decreased fertility.

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4 EFFECTS OF SUPPLEMENTAL PROGESTERONE AFTER AI ON EXPRESSION OF INTERFERON-STIMULATED GENES AND FERTILITY IN DAIRY COWS

Abstract

Objectives were to evaluate the effects of supplemental progesterone after artificial insemination (AI) on expression of interferon-stimulated genes (ISG) in blood leukocytes and fertility in lactating dairy cows. Weekly cohorts of Holstein cows were blocked by parity (575 primiparous and 923 multiparous) and method of insemination as timed AI or AI on estrus and allocated randomly within each block to untreated controls, a controlled-internal drug release (CIDR) containing 1.38 g of progesterone from d 4 to 18 after AI (CIDR4), or a CIDR on d 4 and another on d 7 after AI and both removed on d 18 (CIDR4+7). Blood was sampled to quantify progesterone concentrations in plasma and mRNA expression in of leukocytes for the ubiquitin-like IFN-stimulated gene 15-kDa protein (*ISG15*) and receptor transporter protein-4 (*RTP4*) genes. Pregnancy was diagnosed on d 34 ± 3 and 62 ± 3 after AI. Treatment increased progesterone concentrations between d 5 and 18 after AI in a dose-dependent manner (control = 3.42, CIDR4 = 4.97, and CIDR4+7 = 5.46 ng/mL). Cows supplemented with progesterone tended to have increased luteolysis by d 19 after AI (control = 17.2 vs. CIDR4 = 29.1 vs. CIDR4+7 = 30.2%), which resulted in a shorter AI interval for those reinseminated after study d 18. Pregnancy upregulated expression of ISG in leukocytes on d 19 of gestation, but supplementing progesterone did not increase mRNA abundance for *ISG15* and *RTP4* on d 16 after insemination and tended to reduce mRNA expression on d 19 after AI. For *RTP4* on d 19, the negative effect of supplemental progesterone was observed only in the nonpregnant cows. No overall effect of treatment was observed on P/AI on d 62 after insemination and averaged 28.6, 32.7, and 29.5% for control, CIDR4 and CIDR4+7, respectively. Interestingly, an interaction between level of supplemental progesterone and method of AI was observed for P/AI. For cows receiving exogenous progesterone, the lower supplementation with CIDR4 increased P/AI on d 62 in cows inseminated following timed AI (CIDR4 = 39.2 vs. CIDR4+7 = 27.5%) but, in those inseminated following detection of estrus, the use of a second insert on d 7 resulted in greater P/AI (CIDR4 = 26.9 vs. CIDR4+7 = 31.5%). Pregnancy loss did not differ among treatments. Supplemental progesterone post-AI using a single intravaginal insert on d 4 was beneficial to pregnancy in cows inseminated following timed AI, but incremental progesterone with a second insert on d 7 did not improve fertility of dairy cows.

Key words: dairy cow, interferon-stimulated gene, progesterone, reproduction.

4.1 Introduction

Progesterone is pivotal for successful pregnancy in ruminants (SPENCER et al., 2007), and lactating dairy cows are known to have reduced systemic concentrations of progesterone during diestrus compared with dairy heifers (SARTORI et al., 2004). It is thought that inadequate progesterone concentrations during early development of the conceptus might be one of the reasons for reduced fertility observed in high-producing dairy cows (WILTBANK et

al., 2011), in part because catabolism of steroids increases with increased feed intake associated with high production (PARR et al., 1993; WILTBANK et al., 2011). In fact, supplemental progesterone after AI from an exogenous source (STEVENSON et al., 2007; WILTBANK et al., 2011) resulted in small increases in pregnancy per AI (P/AI). Compared with untreated controls, beef heifers treated with exogenous progesterone starting on d 3 of the estrous cycle had a larger conceptus on d 17 (CARTER et al., 2008, CLEMENTE et al., 2009). Supplementing progesterone post-AI increased concentrations of interferon-tau (IFN- τ) in the uterine lumen (MANN; FRAY; LAMMING, 2006), which reflects the expanded elongation of the trophoderm from the conceptus of cows with increased concentrations of progesterone (MANN; FRAY; LAMMING, 2006; CLEMENTE et al., 2009).

Timed AI protocols result in variable sizes of ovulatory follicles in dairy cows (SOUZA et al., 2007; SANTOS et al., 2010). Induction of ovulation of small follicles resulted in reduced P/AI in beef (PERRY et al., 2005) and dairy cows (SOUZA et al., 2007), and increased risk of pregnancy loss (PERRY et al., 2005). Embryo quality in cows synchronized for timed AI is of equal or better quality than that of cows inseminated after detected estrus (CERRI et al., 2009b); however, cows subjected to timed AI protocols that are induced to ovulate small follicles have a small resulting corpus luteum (CL) with reduced ability to increase peripheral concentrations of progesterone (VASCONCELOS et al., 2001), thereby potentially reducing P/AI (PARR et al., 2012). Therefore, benefits from progesterone supplementation might be greater in cows inseminated following timed AI programs.

Interferon- τ secreted by the trophoderm of the conceptus is the main signal for pregnancy recognition, initiating the process to block the luteolytic cascade and preventing the demise of the CL (MEYER et al., 1995). In the bovine conceptus, mRNA for IFN- τ is detected in trophoblast on d 12 of gestation with peaks occurring between d 15 and 16 (FARIN et al., 1990). Interferon gene mRNA expression in conceptuses is activated with developmental stage of the blastocyst, and progesterone plays a pivotal role in stimulating conceptus development in utero (CLEMENTE et al., 2009). In general, it is thought that stimulation of embryo development by an early rise in progesterone should benefit fertility (STRONGE et al., 2005; PARR et al., 2012), possibly because of advancing conceptus development. Nevertheless, fertility responses to exogenous progesterone seem to be greater when supplementation occurs before (BISINOTTO et al., 2013) than after (STEVENSON et al., 2007) AI. In most studies with post-AI progesterone supplementation to lactating dairy cows no apparent attempt was made to mimic the normal rise in progesterone observed in heifers, which is greater and faster than that of cows (SARTORI et al., 2004). An exception is the recent work by Nascimento et

al. (2013) in which lactating dairy cows receiving an injection of 3,300 IU of human chorionic gonadotropin (hCG) on d 5 concurrent with insertion of a controlled internal drug release containing progesterone had progesterone profiles during diestrus similar to those of dairy heifers. The authors speculated that such manipulation that mimics the progesterone concentrations in heifers might benefit fertility of lactating dairy cows. Therefore, it is possible that the limited benefit to post-AI progesterone supplementation on pregnancy might be the result of insufficient supplementation or inability to mimic the continuous rise and incremental difference during diestrus between groups known to have low (lactating cows) and those of high fertility (heifers).

Interferon- τ binds type I IFN receptor (ROBERTS et al., 1999), which leads to down-regulation of oxytocin receptor expression on superficial glandular and luminal epithelia in sheep (ROBERTS et al., 1999; SPENCER et al., 2007), and this mechanism is thought to be similar among all domestic ruminants. The down-regulation of oxytocin receptors ultimately inhibits pulsatile release of PGF_{2 α} responsible for the demise of the CL (MEYER et al., 1995; SPENCER et al., 2007). Production of IFN- τ by the conceptus induces IFN-stimulated genes (ISG) in the endometrium such as myxovirus (influenza virus) resistance 1 (*Mx1*) and receptor transporter protein 4 (*RTP4*; HICKS et al., 2003; GIFFORD et al., 2008). Blood leukocytes harvested on d 16 or 19 after AI had increased expression of the ISG ubiquitin-like IFN-stimulated gene 15-kDa protein (*IGS15*), *Mx1*, *Mx2*, and *RTP4* (GIFFORD et al., 2007; RIBEIRO et al., 2014), and leukocyte mRNA for ISG was correlated with the amount of IFN- τ in the uterus (MATSUYAMA et al., 2012). Interestingly, stimulation of conceptus development during pre- and peri-implantation resulted in increased expression of ISG in blood leukocytes and increased P/AI in lactating dairy cows (RIBEIRO et al., 2014).

The main hypothesis of the present study was that supplemental progesterone starting during metestrus would improve P/AI in dairy cows, particularly in those synchronized for timed AI. It was thought that supplemental progesterone would increase concentrations of progesterone in plasma in a dose-dependent manner, which would stimulate mRNA abundance for ISG in peripheral blood leukocytes, as a measure of improved embryonic-maternal crosstalk, thereby supporting improved pregnancy. Therefore, the main objective of the present study was to investigate the effects of supplemental progesterone starting during metestrus on P/AI when cows are inseminated following detected estrus or timed AI. Additional objectives were to characterize concentrations of progesterone in plasma, luteal lifespan, and abundance of mRNA for ISG in leukocytes in lactating dairy cows supplemented with progesterone.

4.2 Materials and methods

All procedures involving animals in this study were approved by the University of Florida Non-Regulatory Animal Research Committee.

4.2.1 Cows, Housing and Diets

The study was conducted on a dairy farm in Central Florida milking 5,400 cows with a yearly rolling herd average milk yield of 10,700 kg during the study period. Weekly cohorts of cows were enrolled during 7 consecutive weeks and all inseminations were performed from March 25 to May 9, 2013. Primiparous ($n = 575$) and multiparous ($n = 923$) cows were housed separately in free-stall barns equipped with sprinklers and fans. Cows received the same TMR to meet or exceed the nutrient requirements for a lactating Holstein cow producing 45 kg of milk per d with 3.5% fat and 3.1% true protein when DM intake is 24 kg/d (NRC, 2001). Diet consisted of rye grass silage, corn silage, corn earlage, ground corn, citrus pulp, solvent extracted soybean meal, expeller soybean meal, corn gluten feed, molasses, minerals and vitamins. Cows were fed twice and milked thrice daily.

4.2.2 Sample Size and Experimental Design

The sample size was calculated using the MINITAB statistical software ver. 16 using a one-sided test to provide sufficient experimental units to detect statistical significance ($\alpha = 0.05$; $\beta = 0.20$) when P/AI increased 6 percentage units (e.g. 30 vs. 36%) with supplemental progesterone. Approximately 488 cows per treatment were deemed necessary, or a total of 1,464. Because of potential attrition during the study, a total of 1,498 were randomly assigned to treatments in a randomized complete block design. Two blocking criteria were used before randomization to treatments, method of AI, as detected-estrus or timed AI, and parity, as primiparous or multiparous. Four randomization forms were pre-prepared, one for each combination of blocks: primiparous inseminated at detected estrus, primiparous inseminated at fixed time, multiparous inseminated at detected estrus, and multiparous inseminated at fixed time. Within each form, treatments were randomized within a block containing three cows such that each block had each of the three treatments represented. Cows were enrolled in sequence

of availability as they were found in the farm on d 4 after AI in one of the four forms according to the blocking criteria, and day of insemination was considered study d 0.

4.2.3 Treatments and Reproductive Program

Treatments were no supplemental progesterone (Control; n = 499), a single controlled internal drug release (CIDR; Eazi-Breed CIDR Cattle Insert; Zoetis, Madison, NJ) insert containing 1.38 g of progesterone administered on d 4 (CIDR4; n = 504) after AI and retained until d 18 after AI, or two CIDR, with one administered on d 4 and another one on d 7 after AI (CIDR4+7; n = 495) and both retained until d 18 after AI (Figure 4.1).

At first AI, all cows underwent a presynchronization program receiving two treatments of 25 mg of PGF_{2α} each (5 mL of Lutalyse sterile solution; 5 mg/mL of dinoprost as tromethamine salt, Zoetis) on d 43 ± 3 and 57 ± 3 postpartum. After the second PGF_{2α}, cows were eligible to be inseminated if detected in estrus. Cows not identified in estrus by d 69 ± 3 postpartum were enrolled in a 5-d timed AI protocol (d 69 GnRH [2 mL of Cystorelin; gonadorelin diacetate tetrahydrate equivalent to 43 µg of gonadorelin/mL, Merial Ltd., Duluth, GA], d 74 and 75 PGF_{2α}, d 77 GnRH and timed AI). All cows not returning to estrus spontaneously received in advance an injection of GnRH injection for pre-enrollment for resynchronization on d 29 ± 3 after AI. Cows diagnosed non-pregnant on d 34 ± 3 after AI resumed the 5-d timed AI protocol, and timed AI was performed on d 37 ± 3 d after the previous insemination. Throughout the study, after 57 DIM, cows had their tailheads painted using paintsticks (All-Weather Paintstik; LA-CO Industries Inc., Chicago, IL), and detection of estrus was evaluated daily, in the morning, based on removal of the tail paint. Cows identified in estrus were inseminated on the same morning.

4.2.4 Blood Sampling and Analysis of Progesterone in Plasma

Blood was sampled from a subset of 20 randomly selected blocks of cows (60 cows), 20 controls, 20 CIDR4, and 20 CIDR4+7 on study d 4, immediately before progesterone administration, and then again in the mornings of d 5, 7, immediately before placement of the second progesterone insert in CIDR4+7, 8, 11, 14, 16, 18 and 19. A second subset of 60 randomly selected blocks of cows were also sampled, 60 controls, 60 CIDR4, and 60 CIDR4+7 on study d 8, 16, and 19.

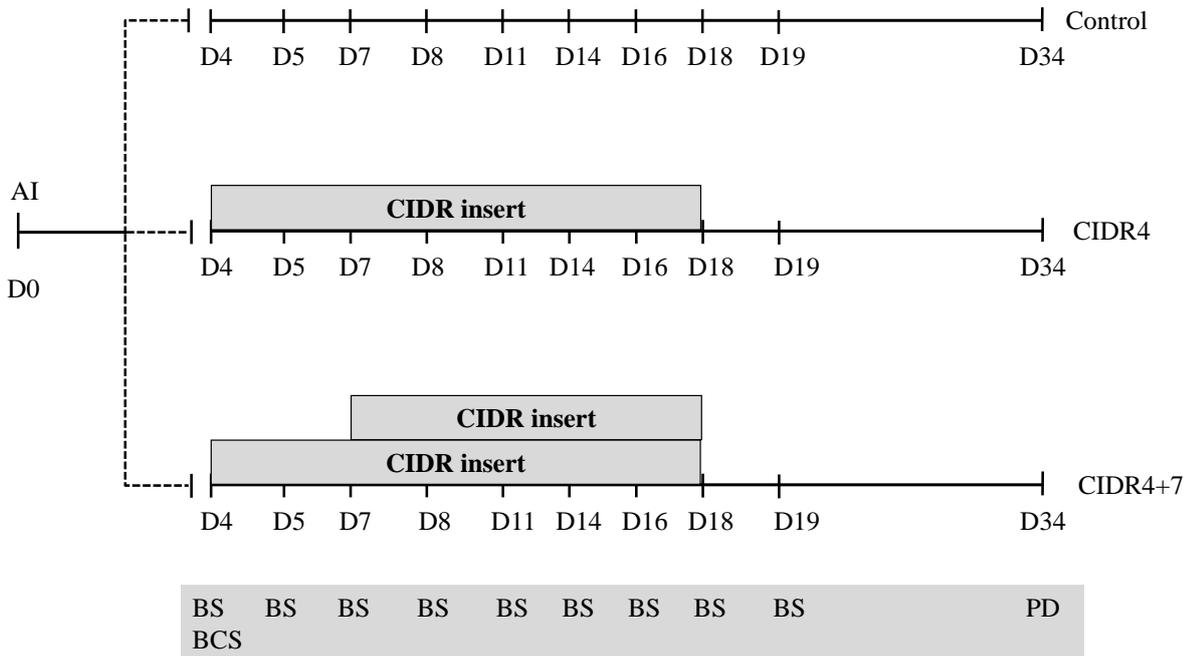


Figure 4.1 – Diagram of activities for the study. Study day 0 is the day of insemination. Treatments were Control, cows received no supplemental progesterone ($n = 492$); CIDR4, cows received a controlled internal drug-release (CIDR) insert containing progesterone from d 4 to 18 ($n = 492$); and CIDR4+7, cows received a controlled internal drug-release insert containing progesterone on d 4 and another on d 7 and they were both removed on d 18 ($n = 484$). AI = artificial insemination; BCS = body condition scoring; BS = blood sampled from a subset of 80 blocks of cows ($n = 240$) and analyzed for progesterone concentrations on d 8, 16, and 19 and leukocytes isolated and mRNA quantified for interferon-stimulated genes on d 16 and 19. Another subset of 20 blocks of cows ($n = 60$) had blood sampled from d 4 to 19. PD = pregnancy diagnosis

Blood was sampled by puncture of the coccygeal vein or artery into evacuated tubes containing K_2 EDTA (Vacutainer; Becton Dickinson, Franklin Lakes, NJ). Immediately upon collection, tubes with blood were placed in ice and kept refrigerated until transported to the laboratory within 4 to 5 h for processing. Blood tubes were centrifuged at $1,500 \times g$ for 15 min at 4°C for plasma separation. Aliquots of plasma were frozen at -20°C until assayed. Concentrations of progesterone were analyzed in plasma by RIA using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). Three assays were performed and the sensitivity of assays 1, 2 and 3 was at least 0.05 ng/mL when calculated as 2 SD below the mean counts per minute at maximum binding. Samples with low and moderate concentrations of progesterone, 1.3 and 4.0 ng/mL , respectively, were incorporated into each assay multiple times for quality control and for calculation of intra and inter-assay CV. The intra-assay CV for the low and moderate progesterone samples were, respectively, 8.3 and 3.5% in assay 1, 4.7 and 5.1% in assay 2, and 3.7 and 4.0% in assay 3. The inter-assay CV for the low and moderate samples were 1.1 and 1.4% , respectively.

4.2.5 Leukocyte Isolation and mRNA Extraction

Blood sampled on d 16 and 19 from 58 blocks of cows from which progesterone concentrations were measured were also used for leukocyte isolation as described by Ribeiro et al. (2014). The pellets of isolated leukocytes were suspended with 0.8 mL of Trizol, transferred to microcentrifuge tubes and stored at -80°C until RNA extraction.

On the day of RNA extraction, samples were removed from -80°C freezer and 200 µL of chloroform were added for each 1 mL of solution to reach a final concentration of 20%. The microcentrifuge tubes were homogenized vigorously by hand for 15 sec and incubated at room temperature for 3 min. Tubes were centrifuged at 12,000 x *g* for 15 min at 4°C for removal of the upper aqueous solution containing RNA. Subsequent RNA extraction was performed using a commercial kit (Purelink RNA Mini Kit, Cat. No. 12183018A, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

4.2.6 Real Time qPCR

Isolated RNA was evaluated for concentration and purity using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Rockford, IL). Subsequently, the samples were incubated with DNAase (DNAase I, Cat. No.M0303, New England BioLabs Inc., Ipswich, MA) for 30 min at 37°C to remove genomic DNA and subsequently heat-denatured at 75°C for 15 min. A total of 250 ng of RNA was reverse transcribed to complementary DNA using a commercial kit (High-capacity cDNA Reverse Transcription Kit, Cat. No. 4368813, Applied Biosystems, Foster City, CA) following manufacturer's instructions. Real time qPCR was performed using SYBR Green PCR Master Mix (Cat. No. 4385614, Applied Biosystems) and the ABI 7300 Real Time PCR System (Applied Biosystems). After an initial activation at 60°C for 2 min followed by denaturation at 95°C for 10 min, the amplification protocol followed 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each sample was evaluated in triplicate, and the specificity for amplification was verified by melting curve analysis. Four genes were investigated (Table 4.1), including the two reference genes, beta-actin (*ACTB*) and ribosomal protein L19 (*RPL19*), and two target genes, *ISG15* and *RTP4*.

Table 4.1 – Gene, primer orientation, primer sequence (5' to 3'), and National Center for Biotechnology Information (NCBI) accession number and sequence for primers used in RT-qPCR assays

Gene	Primer	Sequence (5' to 3')	NCBI sequence
<i>ACTB</i>	Forward	CTGGACTTCGAGCAGGAGAT	AY141970
	Reverse	GATGTCGACGTCACACTTC	
<i>ISG15</i>	Forward	GGTATCCGAGCTGAAGCAGTT	NM_174366
	Reverse	ACCTCCCTGCTGTCAAGGT	
<i>RPL19</i>	Forward	ATTGACCGCCACATGTATCA	NM_001040516
	Reverse	GCGTGCTTCCTTGGTCTTAG	
<i>RTP4</i>	Forward	TTCTCCCCAGAAAGCAGCAA	BC105539
	Reverse	TTCACAGTTGGCCTTGTCATG	

4.2.7 Pregnancy Diagnosis and Calculation of Reproductive Responses

Pregnancy was diagnosed by transrectal ultrasonography on d 34 ± 3 after AI. The presence of an amniotic vesicle containing an embryo with heartbeat was used as determinant of pregnancy. Pregnant cows on d 34 were re-examined for pregnancy by transrectal palpation 4 wk later, on d 62 ± 3 of gestation. Pregnancy per AI was calculated by dividing the number of cows diagnosed pregnant at 34 ± 3 or 62 ± 3 d after AI by the number of cows receiving AI. Pregnancy loss was calculated as the number of cows that lost a pregnancy between d 34 ± 3 and 62 ± 3 after AI divided by the number of cows diagnosed pregnant on d 34 ± 3 after AI. Cows that were detected in estrus before pregnancy diagnosis were re-inseminated and considered as non-pregnant.

4.2.8 Body Condition Score and Milk Yield

The body condition of all cows was scored on study d 4 according to Ferguson et al. (1994) using the Elanco BCS chart (Elanco, 2009). For statistical analysis, BCS was categorized as low, when $BCS \leq 2.75$, or moderate, when $BCS \geq 3.00$. Yields of milk were recorded for individual cows once monthly using on-farm milk meters (Tru-Test Ltd., Manukau, New Zealand). The production on the month of insemination was categorized as above or below the mean milk yield within primiparous and within multiparous cows in the study and were included in the statistical models for data analyses.

4.2.9 Statistical Analysis

Categorical data were analyzed by logistic regression using the GLIMMIX procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC) fitting a binary distribution. The models included the fixed effects of treatment, parity, type of AI, BCS category, number of AI (first AI vs. resynchronized AI), categorized milk yield within parity in the month of AI as above or below the mean value, and the interactions between treatment and parity, treatment and type of AI, and treatment and number of AI, and the random effect of block. For P/AI and pregnancy loss, the fixed effects of sire and technician were also included in the models. The Kenward-Roger method was used to calculate the denominator degrees of freedom to approximate the F tests in the mixed models. Model fitting was evaluated using the fit statistics. The estimates were back-transformed using the ILINK function of SAS to generate the adjusted proportions. An additional analysis of pregnancy on d 34 was performed in the 240 cows with progesterone concentration on d 8 after AI to model the effect of progesterone concentration on P/AI. The model included the fixed effects of treatment, progesterone concentration on d 8 as a linear or quadratic term, and the random effect of block. The logistic function was used to model the probability of pregnancy as a function of progesterone concentration.

Continuous data with repeated measures over time were analyzed using the GLIMMIX procedure of SAS with models fitting a Gaussian distribution. Data were tested for normality of residuals, and data with residuals not normally distributed were transformed before analysis. The models included the fixed effects of treatment, day of measurement, parity, type of AI, interactions between treatment and day, treatment and parity, treatment and type of AI, and treatment and number of AI, and the random effects of cows nested within treatment and block. The effect of pregnancy on d 34 and the interaction between treatment and pregnancy on d 34 were also included for the analysis of progesterone concentrations in plasma. When the F-test for an interaction was significant, means were then partitioned using the SLICE command in SAS. The covariance structure that resulted in the smallest Akaike's information criterion was selected for the model. When time intervals between measurements were unequal, then the spatial power covariance structure was used. Model fitting was evaluated using the fit statistics.

Quantitative PCR data are presented using the comparative method developed by Livak and Schmittgen (2001) using nonpregnant cows from the control group as the reference for relative expression of mRNA abundance, which was set to the relative value of 1. The delta cycle threshold (ΔC_T) values for each target gene were obtained after normalization of C_T value

of the gene with the geometric mean of C_T values from the two reference genes according to Vandesompele et al. (2002). Data were analyzed using the ΔC_T for d 16 or d 19 with the Mixed procedure of SAS fitting a model with the fixed effects of treatment, pregnancy on d 34, and the interaction between treatment and pregnancy on d 34, and the random effect of block. The $\Delta\Delta C_T$ were obtained from ΔC_T LSM differences of pairwise comparisons among treatments and the reference group control nonpregnant cows (YUAN et al., 2006). The relative expression values were obtained by raising the PCR amplification efficiency ($E = 2$) to the power $\Delta\Delta C_T$ (YUAN et al., 2006). Confidence limits for graphical representation of relative expression were generated from the lower and upper CI obtained for ΔC_T LSM differences as described by Yuan et al. (2006).

Orthogonal comparisons were used to determine the effects of supplementing progesterone with CIDR (control vs. CIDR4 + CIDR4+7) and the effects of amount of progesterone supplemented (CIDR4 vs. CIDR4+7). Contrasts for the interactions between type of AI and supplemental progesterone or the amount of supplemental progesterone were also tested. Differences with $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ were considered tendencies.

4.3 Results

Of the 504 CIDR4 cows, 19 lost the insert before d 18 of the study (3.8%). Of the 495 CIDR4+7 cows, 28 lost at least 1 insert (5.6%), of which 20 lost a single insert (4.0%) and 8 lost both inserts (1.6%) before d 18. Twelve of the 1,498 initially enrolled cows were excluded from the data analyses (4 control, 4 CIDR4, 4 CIDR4+7) because of errors during treatment administration or because they received another AI on study d 4, concurrent with treatment administration. Another 18 cows were excluded from the analysis of P/AI because they either died or were sold before the day of pregnancy diagnosis. Therefore, of the initial 1,498 cows, 1,468 were used for statistical analyses of the data.

Milk yield on the month of enrollment did not differ ($P = 0.47$) among treatments and averaged 38.3 ± 0.5 , 39.0 ± 0.5 , and 38.2 ± 0.5 kg/d for control, CIDR4, and CIDR4+7, respectively. Multiparous cows had greater ($P < 0.01$) milk production than primiparous cows (41.9 ± 0.4 vs. 35.1 ± 0.5 kg/d). No difference in milk yield was observed for cows inseminated in estrus or timed AI, and averaged 38.5 kg/d. The DIM at AI for all cows in the study did not differ ($P = 0.66$) among treatments and averaged 114.7 ± 2.1 , 114.9 ± 2.1 , and 117.0 ± 2.1 for control, CIDR4, and CIDR4+7. The proportions of control, CIDR4, and CIDR4+7 cows

receiving first and resynchronized AI, respectively, were 32.5 and 67.5, 35.5 and 64.5, and 34.7 and 65.4%. The mean and median numbers of AI for cows enrolled in the study were 2.7 ± 0.1 and 2.0, and both did not differ among treatments. The median BCS of cows in the study was less ($P = 0.04$) for control than CIDR4 and CIDR4+7 (2.75 vs. 3.00 vs. 3.00). Because of this difference in median BCS, a tendency ($P = 0.08$) was observed for more control cows to have BCS < 3.00 compared with cows receiving CIDR4 and CIDR4+7 (50.7 vs. 43.9 vs. 46.8%).

4.3.1 Concentrations of Progesterone and Luteolysis by d 19

Mean concentrations of progesterone in the 20 blocks of cows sampled throughout the treatment period increased ($P < 0.02$) with supplemental progesterone, but only a numerical increase was observed between means for the CIDR4 and CIDR4+7 (Figure 4.2). As anticipated, concentrations of progesterone did not differ among treatments on d 4. Inclusion of a CIDR in CIDR4 and CIDR4+7 on d 4 after AI increased concentrations of progesterone by approximately 2.2 ng/mL on d 5, 1 d after treatment. The inclusion of a second progesterone insert in CIDR4+7 on d 7 resulted in an additional increment in progesterone concentrations of approximately 1.2 ng/mL on d 8 compared with CIDR4. Concentrations of progesterone increased ($P < 0.001$) with day after AI until it reached a peak on d 16, after which it slightly declined, particularly in the progesterone-supplemented cows. No difference in progesterone concentrations was observed among treatments after d 16 of the study. Milk yield or BCS were not associated with concentrations of progesterone between d 5 and 18 after AI.

From the subset of 240 cows sampled for blood on d 8, 16 and 19, effects of treatment ($P = 0.002$), day ($P < 0.001$), interaction between treatment and day ($P < 0.001$), and pregnancy ($P < 0.001$) were observed for concentrations progesterone (Figure 4.3). For cows eventually diagnosed pregnancy on d 34 (Figure 4.3A), concentrations in plasma increased ($P < 0.05$) with supplemental progesterone and with level of supplemental progesterone on d 8 and 16 of gestation. By d 19, after removal of the CIDR, no differences in progesterone concentrations were observed according to treatments. However, for cows eventually diagnosed as nonpregnant on d 34 (Figure 4.3B), concentrations in plasma increased ($P < 0.05$) with supplemental progesterone and with level of supplemental progesterone only on d 8 after AI. On d 19, after removal of the intravaginal inserts, concentrations of progesterone were less for cows receiving CIDR4 and CIDR4+7 compared with controls. Milk yield or BCS were not associated with concentrations of progesterone on d 8, 16 or 19 after AI.

Supplementing progesterone tended ($P = 0.07$) to increase the incidence of luteolysis on d 19 after AI (Table 4.2). In fact, from all nonpregnant cows on d 34, a greater ($P = 0.01$) proportion of progesterone-supplemented cows had undergone luteolysis by d 19. Level of progesterone supplementation did not affect the risk of luteolysis by d 19 after AI. Method of AI or interaction between treatment and method of AI did not influence the risk of luteal regression by d 19 (Table 4.3).

4.3.2 Interferon-Stimulated Genes Expression in Leukocytes

One CIDR4+7 cow was removed from the analyses of mRNA abundance for ISG in leukocytes because of incorrect administration of treatment. Of the 173 remaining cows, the number of nonpregnant and pregnant cows on d 34 were, respectively, 36 and 22 for controls, 19 and 39 for CIDR4, and 36 and 21 for CIDR4+7.

On d 16 after AI, mRNA abundance for *ISG15* was not influenced by supplemental progesterone ($P = 0.68$), level of progesterone supplementation ($P = 0.29$), pregnancy status on d 34 ($P = 0.41$), or interactions between progesterone treatments and pregnancy status (Figure 4.4, panel A). On the same day, mRNA abundance for *RTP4* was not influenced by supplemental progesterone ($P = 0.17$) or level of progesterone supplementation ($P = 0.66$), but it tended to be less ($P = 0.06$) for pregnant than nonpregnant cows (Figure 4.4, panel B). No interaction was observed between progesterone and pregnancy ($P = 0.45$) or level of progesterone supplementation and pregnancy ($P = 0.54$).

On d 19 after AI, mRNA abundance for *ISG15* tended ($P = 0.10$) to be less for cows supplemented with progesterone than controls, but no difference was observed with level of supplemental progesterone ($P = 0.98$; Figure 4.4, panel C). Pregnant cows on d 34 had greater ($P < 0.001$) mRNA abundance for *ISG15* than nonpregnant cows. No interaction was observed between progesterone supplementation and pregnancy status on d 34 ($P = 0.43$) or level of progesterone supplementation and pregnancy ($P = 0.43$). On the same day, mRNA abundance for *RTP4* increased ($P < 0.001$) in pregnant cows, and it was affected by an interaction ($P < 0.02$) between progesterone supplementation and pregnancy status on d 34 (Figure 4.4, panel D). In pregnant cows, *RTP4* mRNA expression was not influenced ($P = 0.81$) by progesterone supplementation; however, in nonpregnant cows, supplementation with progesterone reduced ($P < 0.01$) *RTP4* gene expression. Level of progesterone supplementation ($P = 0.78$) or interaction between level of progesterone and pregnancy status ($P = 0.70$) did not influence *RTP4* gene expression in leukocytes on d 19.

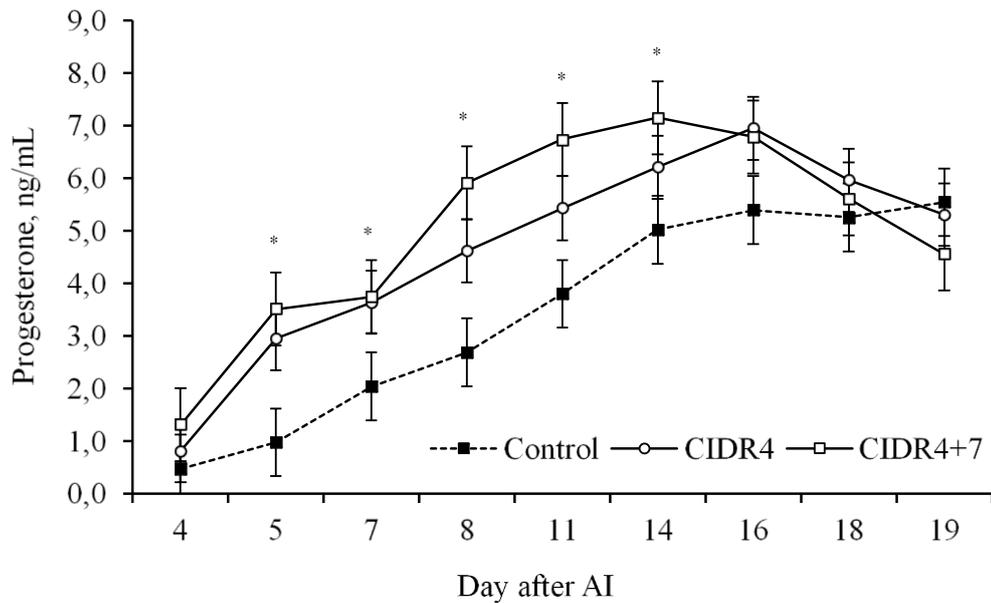


Figure 4.2 – Concentrations of progesterone in plasma according to day after AI. Control, cows received no supplemental progesterone ($n = 20$); CIDR4, cows received a controlled internal drug-release insert containing progesterone from d 4 to 18 ($n = 20$); CIDR4+7, cows received a controlled internal drug-release insert containing progesterone on d 4 and another on d 7 and they were both removed on d 18 ($n = 19$). From d 5 to 18, concentrations averaged 3.42 ± 0.59 , 4.97 ± 0.53 , and 5.46 ± 0.64 ng/mL for control, CIDR4, and CIDR4+7, respectively. Effects of treatment ($P = 0.05$), day ($P < 0.001$), and interaction between treatment and day ($P < 0.01$). Orthogonal comparisons for the effect of supplementing progesterone ($P = 0.02$), and of level of progesterone supplementation ($P = 0.56$). *Within a day, effect of supplemental progesterone ($P < 0.05$)

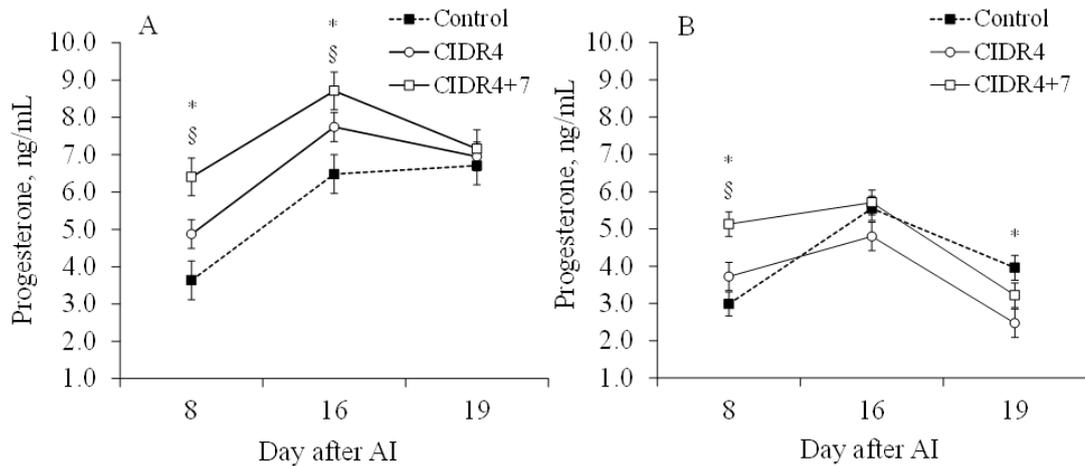


Figure 4.3 – Concentrations of progesterone in plasma of pregnant (A) and nonpregnant (B) cows used for leukocyte isolation and mRNA for interferon-stimulated gene expression ($n = 240$). Control, cows received no supplemental progesterone ($n = 80$); CIDR4, cows received a controlled internal drug-release insert containing progesterone from d 4 to 18 ($n = 80$); CIDR4+7, cows received a controlled internal drug-release insert containing progesterone on d 4 and another on d 7 and they were both removed on d 18 ($n = 80$). Effects of treatment ($P = 0.002$), day after AI ($P < 0.001$), interaction between treatment and day ($P = 0.001$), and pregnancy ($P < 0.001$). Orthogonal comparisons for the effects of supplementing progesterone ($P = 0.02$) and amount of progesterone supplemented ($P < 0.01$). Within a day, pairwise differences ($P < 0.05$) are represented as follows: * effect of progesterone supplementation; § effect of level of progesterone supplementation

4.3.3 Re-insemination in Estrus, Pregnancy per AI, and Pregnancy Loss

The proportion of cows reinseminated on estrus before pregnancy diagnosis on d 34 was not affected by supplemental progesterone, but it was greater ($P = 0.04$) for CIDR4+7 than CIDR4 (Table 4.2). When all nonpregnant cows were considered, including those reinseminated on or before d 18, then the interval between pre-enrollment and post-enrollment AI tended ($P = 0.06$) to be longer for cows supplemented with progesterone, but there was no effect of level of progesterone supplementation. With the exception of 3 cows in CIDR4 that returned to estrus on or before d 18, the CIDR, as expected, prevented cows from returning to estrus until the removal of the inserts (Figure 4.5). One cow in CIDR4 lost the CIDR and returned to estrus on d 15, whereas the other two had the CIDR when detected in estrus, one on d 13 and another on d 18. Interestingly, when only cows inseminated after d 18 were considered, which coincided with the removal of the intravaginal inserts in CIDR4 and CIDR4+7, then interval between AI was longer ($P = 0.02$) for control cows than for cows supplemented with progesterone.

Pregnancy per AI on d 34 and 62 after insemination did not differ with supplemental progesterone or level of supplementation (Table 4.2). However, an interaction ($P < 0.02$) between level of supplemental progesterone and method of AI was observed for P/AI on d 34 and 62 (Table 4.3). For cows inseminated following detection of estrus, P/AI increased with administration of two intravaginal inserts in CIDR4+7 compared with CIDR4; however, for those inseminated following timed AI, administering a single insert with CIDR4 increased P/AI. For cows inseminated following timed AI, treatment with a single progesterone insert improved P/AI compared with control or CIDR4+7 (Table 4.3). When data were analyzed with the 240 cows in which plasma was quantified for concentrations of progesterone on d 8 after AI, a quadratic relationship ($P = 0.08$) was observed between progesterone concentration and the probability of pregnancy on d 34. The same analysis was performed separately for cows inseminated after detected estrus or following timed AI (Figure 4.6). For cows inseminated at detected estrus, the relationship was quadratic ($P = 0.09$); however, for cows receiving timed AI the same relationship was linear ($P < 0.001$). Milk yield or BCS were not associated with P/AI on d 34 or 62 after insemination.

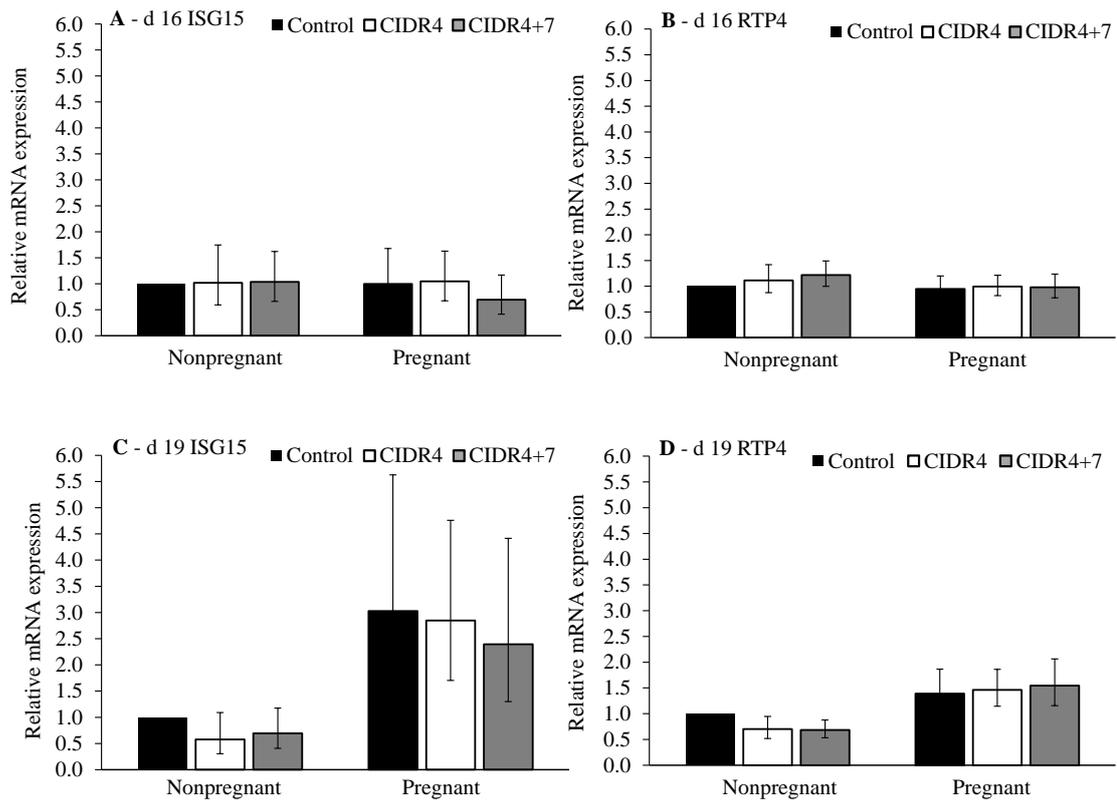


Figure 4.4—Relative abundance and 95% CI of mRNA for interferon-stimulated genes (ISG) in leukocytes isolated from cows receiving no supplemental progesterone (control; 36 nonpregnant and 22 pregnant on d 34), a controlled internal drug-release insert containing progesterone from d 4 to 18 (CIDR4; 19 nonpregnant and 39 pregnant on d 34), or a controlled internal drug-release insert containing progesterone on d 4 and another on d 7 and they were both removed on d 18 (CIDR4+7; 36 nonpregnant and 21 pregnant on d 34). Control nonpregnant cows were the reference group for depicting relative mRNA expression. Panel A, *ISG15* expression on d 16 after AI. Panel B, receptor transporter protein-4 (*RTP4*) expression on d 16 after AI. Panel C, *ISG15* expression on d 19 after AI. Panel D, *RTP4* expression on d 19 after AI. For Panel B, effect of pregnancy ($P = 0.06$). For panel C, effect of supplemental progesterone ($P = 0.10$) and of pregnancy ($P < 0.001$). For panel D, effect of supplemental progesterone ($P < 0.10$), pregnancy ($P < 0.001$), and interaction between supplemental progesterone and pregnancy ($P < 0.02$)

Pregnancy loss between d 34 and 62 of gestation was not influenced by supplemental progesterone or by the amount of progesterone supplemented to cows (Table 4.2). Similarly, no interaction was observed between method of AI and supplemental progesterone or amount of progesterone supplemented for pregnancy loss (Table 4.3). Cows inseminated following detection of estrus tended ($P = 0.10$) to have increased pregnancy loss compared with those inseminated following timed AI (10.0 vs. 5.7%). Milk yield or BCS were not associated with pregnancy loss between gestation d 34 and 62.

Table 4.2 – Effect of the supplemental progesterone (P4) after AI on fertility responses in lactating dairy cows

	Treatment ¹			P ²		
	Control	CIDR4	CIDR4+7	TRT	P4	Level P4
Adjusted proportions (n/n) or LSM (\pm SEM)						
Luteolysis by d 19, ³ %						
All cows	17.2 (11/71)	29.1 (21/78)	30.2 (28/79)	0.23	0.09	0.91
Nonpregnant cows	23.7 (11/49)	62.5 (21/39)	45.2 (28/58)	0.01	< 0.01	0.21
Reinsemination in estrus						
Reinseminated, ⁴ %	59.1 (195/335)	54.6 (170/319)	63.0 (197/321)	0.13	0.94	0.04
Day of reinsemination all days ⁵	21.9 \pm 0.34	22.4 \pm 0.36	22.9 \pm 0.33	0.10	0.06	0.37
Day of reinsemination after d 18 ⁶	23.6 \pm 0.29	22.8 \pm 0.29	22.9 \pm 0.26	0.06	0.02	0.94
Pregnant, %						
Day 34	30.8 (154/492)	35.2 (172/492)	33.2 (161/484)	0.40	0.23	0.55
Day 62	28.6 (142/492)	32.7 (161/492)	29.5 (145/484)	0.39	0.37	0.31
Pregnancy loss, ⁷ %	6.5 (12/154)	6.4 (11/172)	10.2 (16/161)	0.41	0.58	0.25

¹ Control, cows received no supplemental progesterone; CIDR4, cows received a controlled internal drug-release insert containing progesterone from d 4 to 18; and CIDR4+7, cows received a controlled internal drug-release insert containing progesterone on d 4 and another on d 7 and they were both removed on d 18

² TRT = effect of treatment; P4 = orthogonal comparison for the effect of supplemental progesterone (control vs. CIDR4 + CIDR4+7); Level P4 = orthogonal comparison for level of supplemental progesterone (CIDR4 vs. CIDR4+7)

³ The proportion of cows sampled for blood on d 19 that had progesterone < 1 ng/mL

⁴ The proportion of nonpregnant cows reinseminated by AI after detected estrus before pregnancy diagnosis at 34 \pm 3 d after AI

⁵ Interval between AI on study d 0 and reinsemination of cows detected in estrus any day before pregnancy diagnosis on d 34

⁶ Interval between AI on study d 0 and reinsemination of cows detected in estrus after d 18, when CIDR were removed, and before pregnancy diagnosis on d 34

⁷ Pregnancy loss between 34 and 62 d of gestation

Table 4.3 – Effect of supplemental progesterone (P4) on fertility responses according to method of AI¹

	Detected estrus			Timed AI			<i>P</i> ²		
	Control	CIDR4	CIDR4+7	Control	CIDR4	CIDR4+7	AI	P4 x AI	Level P4 x AI
	Adjusted proportions (n/n)								
Luteolysis by d 19, ³ %	22.4 (4/18)	33.3 (7/21)	19.2 (4/20)	13.0 (7/53)	25.2 (14/57)	43.9 (24/59)	0.91	0.20	0.07
Pregnant, %									
Day 34	29.6 ^b (79/252)	30.0 ^b (79/255)	35.7 ^{ab} (86/243)	32.0 ^b (75/240)	40.7 ^a (93/237)	30.9 ^b (75/241)	0.10	0.79	0.01
Day 62	26.0 ^b (70/252)	26.9 ^b (72/255)	31.5 ^b (77/243)	31.3 ^b (72/240)	39.2 ^a (89/237)	27.5 ^b (68/241)	0.31	0.96	0.02
Pregnancy loss, ⁴ %	11.0 (9/79)	9.3 (7/79)	9.8 (9/86)	3.8 (3/75)	4.5 (4/93)	10.6 (7/75)	0.10	0.31	0.30

^{a,b} Values within the same row differ ($P < 0.05$)

¹ All cows were subjected to AI after detected estrus or by timed AI. Control, cows received no supplemental progesterone; CIDR4, cows received a controlled internal drug-release insert containing progesterone from d 4 to 18; and CIDR4+7, cows received a controlled internal drug-release insert containing progesterone on d 4 and another on d 7 and they were both removed on d 18

² AI = effect of method of AI; P4 x AI = interaction between supplemental progesterone (control vs. CIDR4 + CIDR4+7) and AI; Level P4 x AI = interaction between level of supplemental progesterone (CIDR4 vs. CIDR4+7) and AI

³ The proportion of cows sampled for blood on d 19 that had progesterone < 1 ng/mL

⁴ Pregnancy loss between 34 and 62 d of gestation

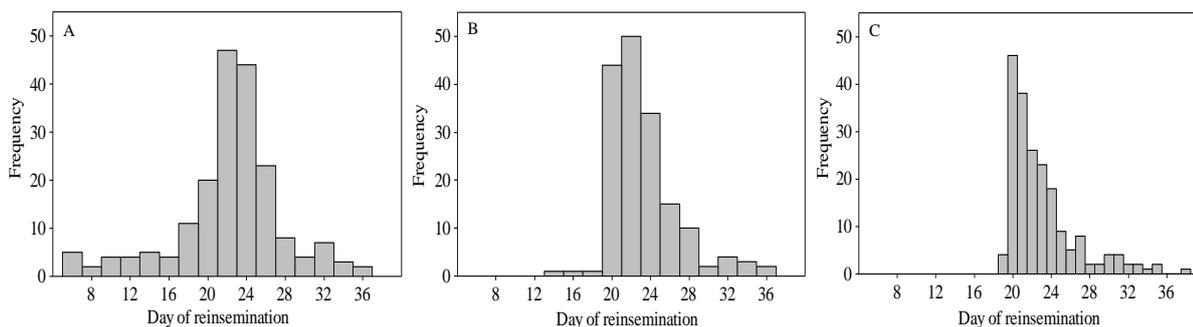


Figure 4.5 – Histogram of day of reinsemination on estrus for control (A; cows received no supplemental progesterone), CIDR4 (B; cows received a controlled internal drug-release insert containing progesterone from d 4 to 18), and CIDR4+7 treatment (C; cows received a controlled internal drug-release insert containing progesterone on d 4 and another on d 7 and they were both removed on d 18)

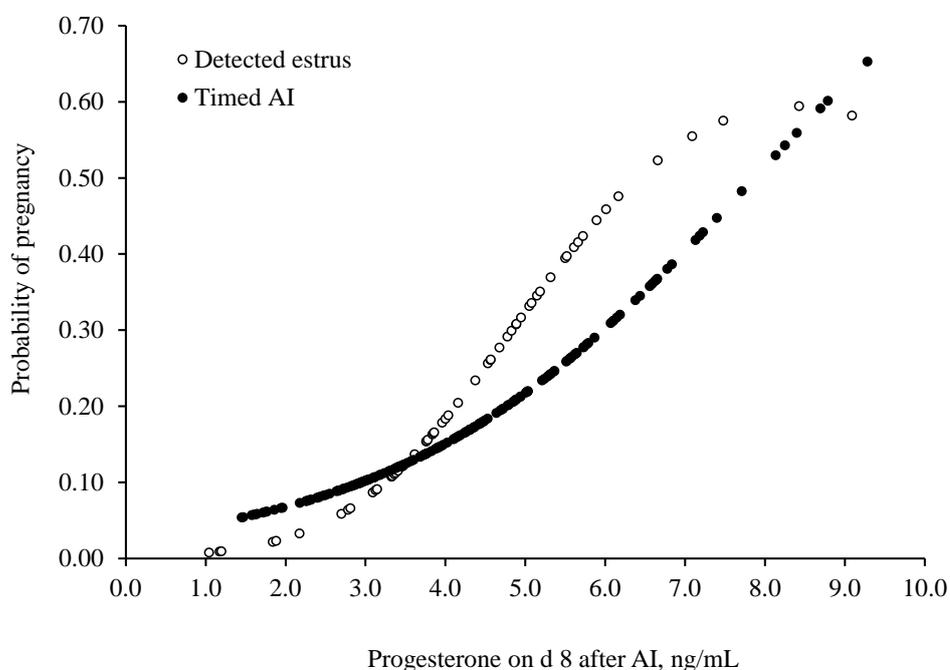


Figure 4.6 – Probability of pregnancy for cows inseminated following detected estrus (\circ ; $n = 63$) or after timed AI (\bullet ; $n = 177$) according to concentration of progesterone in plasma on d 8 after insemination. For cows inseminated upon detected estrus, a quadratic relationship ($P = 0.09$) was observed between concentration of progesterone on d 8 and pregnancy per AI. For cows inseminated following timed AI, the relationship was linear ($P < 0.001$)

4.4 Discussion

The main hypothesis of the present study was that progesterone rise after insemination was an important limiting factor for pregnancy in lactating dairy cows. Supplementing progesterone to mimic concentrations that typically are observed in dairy heifers, which were anticipated to length exposure of the conceptus and endometrium to concentrations compatible

with those observed during the luteal phase, would increase P/AI likely because of stimulation of embryo development. However, supplementing progesterone with intravaginal inserts, which are known to deliver approximately 90 mg/d (RATHBONE et al., 2002) and increase concentrations in plasma by approximately 1 ng/mL (CERRI et al., 2009a), had minor impacts on fertility responses of lactating dairy cows.

An additional hypothesis was that the beneficial effects of supplementing progesterone on fertility would be exacerbated in cows inseminated following timed AI. Although properly implemented timed AI protocols result in good to excellent embryo quality (CERRI et al., 2009b), synchronizing ovulation also results in variable size ovulatory follicles (SOUZA et al., 2007; SANTOS et al., 2010), and inducing ovulation of small follicles reduces concentrations of progesterone during the subsequent diestrus (VASCONCELOS et al., 2001) and P/AI (PERRY et al., 2005; SOUZA et al., 2007). No interaction between supplemental progesterone and method of AI was observed, indicating that exogenous progesterone as CIDR4 and CIDR4+7 did not have a differential effect on pregnancy in cows inseminated either on estrus or following timed AI; however, the single insert on d 4 benefited pregnancy in cows inseminated following timed AI. In fact, when only supplemented cows were considered, an interaction between level of progesterone supplementation and method of AI was observed. Within estrus-detected cows, addition of a second progesterone insert with CIDR4+7 numerically increased P/AI, but for timed AI cows, the single insert in CIDR4 resulted in greater P/AI than using two sequential inserts in CIDR4+7. Perhaps, cows inseminated on detected estrus had less incidence of multiple ovulation and had some benefit from increased progesterone supplementation. On the other hand, 25 to 30% of the cows inseminated following timed AI develop the ovulatory follicle under low concentrations of progesterone (BISINOTTO et al., 2013), which results in more multiple ovulations and, perhaps, less need for larger amounts of supplemental progesterone. Cows inseminated following timed AI or after detected estrus have many distinct physiological differences, including expression of estrus and size of the ovulatory follicle, and it is possible that they are differentially responsive to two methods of progesterone supplementation used in the current study. In general, increments in progesterone concentrations in early diestrus are linked with improved P/AI (PARR et al., 2012; STRONGE et al., 2005). In the current study, when all cows were analyzed together, and a quadratic association between progesterone on d 8 and pregnancy was observed in the current study, as suggested by others in dairy heifers (PARR et al., 2012). Nonetheless, the relationship between progesterone and pregnancy differed slightly when data from cows inseminated on

estrus were analyzed separately from those of cows subjected to fixed time AI. For cows inseminated following estrus, a positive quadratic relationship was observed indicating that as progesterone increased, so did P/AI, but the benefit declined as concentrations became very high. On the other hand, for timed AI cows the relationship was linear indicating that the incremental benefits of increasing progesterone during diestrus to P/AI were similar at all ranges of progesterone concentrations observed.

The rationale for supplementing progesterone starting on d 4 after AI was based on the results from Mann and Lamming (1999) that observed improved P/AI when supplementation initiated during late metestrus or early diestrus, but not after d 6 post-AI. It is thought that lactation, with associated increases in DM intake, enhances the catabolism of progesterone by the splanchnic tissues, primarily the liver (PARR et al. 1993; WILTBANK et al., 2011). A reduction in progesterone could limit proper uterine priming and conceptus development (CARTER et al., 2008; CLEMENTE et al., 2009), thereby reducing P/AI in dairy cows (PARR et al., 2012; STRONGE et al., 2005). Dairy heifers, which are known to have high fertility, have a steeper rise in post-ovulation progesterone concentrations starting on d 4 after AI compared with lactating dairy cows (SARTORI et al., 2004), and the differences in concentrations approximate 2 ng/mL starting on d 7 after AI. Nascimento et al. (2013) demonstrated that concurrent use of a CIDR and treatment with 3,300 IU of hCG on d 5 of the estrous cycle resulted in progesterone profiles in lactating dairy cows that were similar to those of dairy heifers; however, the same authors demonstrated that use of a single CIDR or only hCG on d 5 did not result in progesterone profiles in lactating dairy cows that mimic those of heifers. In the current study, addition of a single intravaginal insert in CIDR4 increased progesterone concentrations by approximately 1.5 ng/mL between d 5 and 18 after AI, and the inclusion of a second insert on d 7 in CIDR4+7 resulted in an additional 0.5 ng/mL increment in progesterone concentration during the same period. When a larger number of cows was evaluated, but only on d 8 and 16 of the study, then progesterone concentrations increased from 4.7 ng/mL in controls to 5.3 in CIDR4 and to 6.5 ng/mL in CIDR4+7. Therefore, the increments in progesterone concentrations with addition of the CIDR were compatible with the findings of others in which each insert results in approximately 0.8 to 1.0 ng/mL additional progesterone in plasma (CERRI et al., 2009a; BISINOTTO et al., 2013). Furthermore, the changes in the present study mimic increases in progesterone associated with higher fertility in heifers (SARTORI et al., 2004).

Overall, P/AI did not differ between controls and cows supplemented with progesterone, despite the positive association between concentrations on d 8 and pregnancy on d 34. It was

thought that supplementing progesterone during metestrus and early diestrus would stimulate endometrial secretion of histotroph as demonstrated by the advancement in global gene expression of endometrium in beef heifers (FORDE et al., 2009). Heifers receiving an intravaginal insert on d 3 of the estrous cycle showed advancements in temporal changes in endometrial gene expression compared with heifers not supplemented with progesterone (FORDE et al., 2009). Concentration of progesterone during early to mid-diestrus was associated with changes in endometrial gland ducts (WANG et al., 2007), that likely increase the supply of nutrients for the developing conceptus and accommodate changes for subsequent placentation (SPENCER et al., 2007). The changes in endometrial gene expression and consequent alterations in glandular function and histotroph supply were thought to mediate the advancements in conceptus elongation and changes in embryonic gene expression when heifers had increased concentrations of progesterone (CARTER et al., 2008, 2010). Despite the links between progesterone concentrations during early diestrus and fertility in dairy cows as observed in the current study and demonstrated by others (PARR et al., 2012), increasing concentrations by almost 2 ng/mL in CIDR4 and CIDR4+7 cows compared with controls did not result in increased P/AI or reduced pregnancy loss. Mann and Lamming (1999) reviewed the literature on progesterone supplementation after AI and observed an average of 5 percentage units increment in P/AI. The same authors indicated that most of the benefit of exogenous progesterone was detected when supplementation initiated before d 6 after AI (MANN; LAMMING, 1999). Interestingly, Stevenson et al. (2007) also demonstrated a 5 percentage units, from 28.3 to 32.7%, increment in P/AI in dairy cows when receiving supplemental progesterone through CIDR inserts, but contrary to the findings of Mann and Lamming (1999), all the benefit occurred when the device was inserted after d 6. In the current study, stimulation of P/AI with exogenous progesterone was only observed when a single insert was administered on d 4 to cows inseminated following timed AI.

In general, increasing progesterone concentrations early in the estrous cycle stimulates conceptus development (MANN; FRAY; LAMMING, 2006; CARTER et al., 2008), which in turn results in increased concentrations of IFN- τ in the uterine lumen (MANN; FRAY; LAMMING, 2006). Interferon- τ produced by the trophoblast cells of the conceptus has local effects on the endometrium, but also effects on cells in other tissues (HICKS et al., 2003; OTT; GIFFORD, 2010). Interferon- τ exits the uterus and reaches the maternal circulation (Oliveira et al., 2008), which induces expression of genes in blood leukocytes (GIFFORD et al., 2008; OLIVEIRA et al., 2008). In fact, mRNA abundance for ISG in leukocytes parallels the

concentrations of IFN- τ in utero (MATSUYAMA et al., 2012). On d 16, expression of ISG in leukocytes was small and indistinguishable between cows diagnosed nonpregnant and pregnant at day 34. This probably reflected the reduced concentrations of IFN- τ in utero at that stage of gestation in cows; however, by d 19, mRNA expression increased substantially in pregnant cows. Nevertheless, exogenous progesterone did not increase mRNA expression for ISG in leukocytes on d 16 and 19 after AI. The lack of positive effect of treatment on ISG in leukocytes might be related to the increase in luteolysis by d 19 in cows that were later found to be nonpregnant. Also, at approximately d 16 of gestation, uterine IFN- τ in bovine peaks and then declines (FARIN et al., 1990). It is possible that the advancements in patterns of endometrial gene expression observed with prolonged exposure to progesterone with supplementation (FORDE et al., 2009) might have also advanced the reduction in mRNA expression of IFN- τ by conceptus trophoblast (FARIN et al., 1999) and, therefore, induced an earlier decline in concentrations in utero.

Two concerns with supplementing progesterone during metestrus are the potential interference with CL formation and function and/or advancement of the luteolytic signals that might induce premature luteolysis. Based on concentrations of progesterone depicted in Figure 4.3A in pregnant cows on d 19, after treatments had ceased, it is plausible to suggest that CIDR4 and CIDR4+7 did not impair the ability of the CL to produce progesterone. Conversely, supplemental progesterone starting during metestrus increased luteolysis on d 19 in nonpregnant cows, which resulted in reduced concentrations of progesterone. These responses in cows later observed nonpregnant are likely explained by the advancements in uterine gene expression observed with prolonged progesterone exposure leading to premature occurrence of luteolysis (LAWSON; CAHILL, 1983). Treatment of ewes with progesterone shortened the estrous cycle by approximately 4 d compared with untreated controls (LAWSON; CAHILL, 1983), which may explain the reductions in pregnancy in heifers supplemented with progesterone within 2 d after AI (VAN CLEEFF et al., 1996). Prolonged exposure to progesterone with supplementation in early diestrus may have advanced the peaks of IFN- τ expression in cows (FARIN et al., 1990; ROBERTS et al., 1999). Increased luteolysis by d 19 in cows later found nonpregnant and potential advancements in peaks of IFN- τ might have precluded the detection of increments in mRNA expression on d 19 for *ISG15* or *RTP4* with progesterone supplementation. Furthermore, it is suggested that reduced pregnancy around the time of conceptus-endometrium cross-talk probably explains the reductions in gene expression of *RTP4* in leukocytes in cows found nonpregnant on d 34.

It is unclear why supplemental progesterone did not result in further stimulation of mRNA expression of ISG in leukocytes of pregnant cows compared with untreated controls. It was thought that pregnant cows would have advanced conceptus development with supplemental progesterone (CARTER et al., 2008), and the latter were expected to have an earlier and more robust increment in expression of ISG because of increased IFN- τ in utero (MATSUYAMA et al., 2012). The mRNA abundance in blood leukocytes of the two genes investigated in the present study were previously described to be associated with improved conceptus development and increased fertility in lactating dairy cows (RIBEIRO et al., 2014). Nonetheless, exogenous progesterone might have attenuated some of the effects of IFN- τ on leukocytes. Addition of progesterone to leukocytes cultured *in vitro* blunted the effect of IFN- α in stimulating mRNA expression for myxovirus resistance protein A (*MxA*; TAYEL et al., 2013), an ISG of the same family as *Mx1* typically quantified in leukocytes of cattle (GIFFORD et al., 2007; RIBEIRO et al., 2014). Interferon- τ and IFN- α are both type I IFN that interact with the same IFN receptor (ROBERTS et al., 1999). Therefore, it is plausible to suggest that the positive effects of increasing systemic progesterone on conceptus development in CIDR4 and CIDR4+7 were not evident in blood leukocytes because of direct immunomodulation of supplemental progesterone on ISG (TAYEL et al., 2013). In fact, responses of blood leukocytes to IFN- τ during early pregnancy are believed to counter-balance the immunosuppressive effects of progesterone on the maternal immune system (OTT; GIFFORD, 2010). A study supplementing somatotropin that stimulated conceptus development, without changes in progesterone concentrations, resulted in increased expression of ISG in leukocytes and P/AI in dairy cows (RIBEIRO et al., 2014). In the present study, if advanced conceptus development was successfully obtained with exogenous progesterone as observed by others (CARTER et al., 2008; CLEMENTE et al., 2009), then the lack of responses in ISG in blood leukocytes and P/AI bring new insights to the interactions among progesterone concentrations, conceptus development, IFN- τ , and maternal immune system during establishment of pregnancy in dairy cattle.

4.5 Conclusion

Supplementing progesterone to lactating dairy cows with intravaginal inserts starting on d 4 after AI increased concentrations of progesterone in plasma in a dose-dependent manner, but did not increase mRNA expression of IFN-induced genes in leukocytes. Although P/AI was

associated with concentrations of progesterone on d 8 after insemination, the use of intravaginal inserts to increase progesterone, up to 2 ng/mL, post-AI did not have an overall beneficial effect on maintenance of pregnancy in lactating dairy cows. When cows were inseminated following timed AI, then a single insert improved P/AI. Furthermore, results indicate that supplemental progesterone during early diestrus increased luteolysis by d 19 in cows found nonpregnant on d 34. Exogenous progesterone supplementation through CIDR was unable to stimulate expression of genes stimulated by interferon, but some benefits were observed in P/AI when a single insert was used in cows inseminated following timed AI.

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5 GENERAL CONCLUSIONS, OTHER STUDY AND FUTURES STUDIES

5.1 General conclusions

The objectives of these studies were to evaluate the hormone supplementation to increase fertility in dairy cows. To improve the response of E2/P4-based FTAI protocol, studies with presynchronization protocols and increasing the EB dose at the beginning of FTAI protocol were carried out. Moreover, it was evaluated the effect of P4 supplementation on AI after estrus detection and in cows subjected to FTAI (E2/P4 or GnRH-based protocols). Finally, the effect of P4 supplementation after in vitro embryo transfer on lactating recipient cows was studied.

Several hypotheses were tested, and the outcomes are presented below:

1. Higher EB dose would increase the synchronization of follicular wave emergence and thereby increase synchronization during the protocol. The EB dose of 2.0 mg would be insufficient, thus could explain the P/AI around 30% on the FTAI E2/P4-based protocols (Souza, Viechnieski, Lima, Silva, Araujo, Bo, Wiltbank, and Baruselli, 2009). By increasing the EB dose to 3.0 mg increased cows with premature CL regression and did not improve synchronization of the follicular wave.
2. Due to the success of presynchronization programs on fertility in GnRH-based protocols (Moreira, Orlandi, Risco, Mattos, Lopes, and Thatcher, 2001; Souza, Ayres, Ferreira, and Wiltbank, 2008), we hypothesized that presynchronization with a single GnRH treatment would synchronize the stage of the follicular wave at the beginning of the E2/P4 protocol and induce a greater synchronization rate. It was observed that, regardless of the follicular wave stage a proportion of cows still failed to have a synchronized emerge a new follicular wave.
3. The most interesting findings of the experiments above were not hypothesized, but when daily ultrasound exams were analyzed, we were able to understand some of the reason that led to lack of synchronization during the protocols. Based on data of emergence of a new follicle wave and ovulation at the end of the protocol, only 60% of the cows were considered to be synchronized. In these cows, the P/AI was about 60%.
4. P4 supplementation post ovulation would not interfere in the CL volume and function. In fact, plasma concentration of P4 was increased between d 4 and 7, and there was no effect on CL volume. Nevertheless, the lifespan of the CL was affected when cows were

supplemented 4 days after AI, therefore a higher proportion of these cows had concentration of P4 on d 19 below 1.0 ng/mL.

5. Cows subjected to P4 supplementation should present increased ISG expression. This hypothesis was rejected due to the fact that ISG expression, in general, was similar between control and treated cows.
6. Supplemental P4 would increase fertility in dairy cows after AI by estrus detection or FTAI protocols. Neither cows inseminated after estrus detection nor by E2/P4-based FTAI protocols had increased P/AI. Nevertheless, cows subjected to GnRH-based FTAI protocol had increased P/AI when supplemented with a single P4 intravaginal device.
7. Progesterone supplementation 4 days before ET would improve P/ET. This hypothesis was rejected, considering that, independent of number of supplementation days (4 or 14 days) lactating recipient cows supplemented with P4 had decreased P/ET.

5.2 Other study

Because the incidence of cows that did not synchronize the emergence of a new follicular wave was about 26.2% and 22.2% failed to ovulate at the end E2/P4-based protocol we hypothesized that GnRH would be better than EB for synchronization of follicular wave emergence. The first reason was because EB-treated cows had premature luteolysis. High circulating P4 during follicle development is important for greater P/AI (BISINOTTO et al., 2013). In our study, pregnant cows had higher P4 concentration during the protocol than non-pregnant cows. The second reason was due to the number of cows that ovulated persistent follicle. Thus, GnRH must be used to improve the follicular wave emergence rate. In relation to ovulation inductor, ECP has been widely used in Brazil, nevertheless, it has longer half-life and lower peak of E2 than EB (SOUZA et al., 2005). Additionally, EB has serum E2 profile more similar to physiology than ECP (SOUZA et al., 2005).

Based on these findings, we performed an applied manipulative study that is not part of this thesis document. In order to increase the P/AI in cows subjected to FTAI protocols which consisted of EB or GnRH at the beginning of protocol associated with P4 intravaginal device (d -10) to synchronize wave emergence. Cows were treated with PGF on d -3 and d -2. The P4 device was removed with the second dose of PGF. Ovulation was induced with ECP on d -2 or EB on d -1. All cows were inseminated on d 0. A total of 418 cows was used in a 2 x 2 factorial design. In this study, when compared EB and GnRH, it was observed that more cows treated with EB on d -10 had greater proportion of luteolysis between d -10 and d -3 ($49.5\% \pm 5.04$

[58/114] vs. $25.8\% \pm 3.98$ [41/146], $P < 0.001$), and smaller number of cows with CL on d -3 ($43.0\% \pm 3.84$ [86/193] vs. $71.4\% \pm 3.31$ [157/517], $P < 0.001$). Consequently, it was observed lower P4 plasma concentration at the PGF day (d -3), in the cows treated with EB than GnRH (1.8 ± 0.31 vs. 3.2 ± 0.22 ng/mL, $P < 0.001$). Cows treated with EB on d -10 had a smaller ovulatory follicle on d 0 ($13.9 \text{ mm} \pm 0.35$ vs. $15.0 \text{ mm} \pm 0.34$, $P = 0.02$). Although cows treated with GnRH provided greater circulating P4 during the protocol, there was no effect of treatment on P/AI at d 28 ($31.2\% \pm 3.54$ [59/197] vs. $34.9\% \pm 3.48$ [74/221], $P = 0.43$, for EB vs GnRH, respectively), or d 56 ($27.0\% \pm 3.41$ [50/197] vs. $32.4\% \pm 3.44$ [67/220], $P = 0.24$, for EB vs GnRH, respectively), and on pregnancy loss ($11.7\% \pm 4.64$ [9/59] vs $5.6\% \pm 2.79$ [6/73], $P = 0.16$, for EB vs GnRH, respectively). With regard to induction of ovulation, there was no effect neither on ovulatory follicle size nor on fertility. The P/AI on d 28 ($31.8\% \pm 3.64$ [59/195] vs. $34.3\% \pm 3.34$ [74/223], $P = 0.59$, for ECP vs EB, respectively), or d 56 ($27.7\% \pm 3.52$ [50/194] vs. $31.6\% \pm 3.34$ [67/223], $P = 0.41$; for ECP vs EB, respectively), and pregnancy loss ($9.9\% \pm 4.26$ [7/74] vs. $6.7\% \pm 3.15$ [8/58], $P = 0.46$; for ECP vs EB, respectively) were similar between treatments. There was no interaction ($P > 0.10$) between the treatments on d -10 and ovulation induction. Although GnRH on d 0 provided better results than EB, as increased circulating P4 and increased ovulatory follicle size, there was no increase on fertility. Maybe if GnRH had higher ovulation rate, considering that only 29% of cows ovulated, could the outcomes might have been different.

5.3 Future studies

Although the results of the experiments did not contribute directly to increase fertility in dairy cows, they signal a pathway for that. Thus, based on the deficiencies identified on the E2/P4-based FTAI protocols, studies can be done in order to understand the possible reasons of some cows not emerging a new follicular wave. Moreover, further studies must be performed to evaluate the effect of P4 supplementation associated with antiluteolytic strategies delaying luteolysis in order to improve maternal recognition of pregnancy.

In cows, when combining E2 with P4, normally it is observed the emergence of a new follicular wave (BO et al., 1993). According to our studies, chapter 1, some cows did not emerge a new wave. Increase of EB dose to 3.0 mg, also showed the same problem. Thus, it is concluded that this finding was not caused by low E2. Probably, the problem is associated with the P4

concentration. FTAI protocols that ensure high concentration of P4 on the beginning of the protocol associated with EB may resolve this problem.

Another study can be done to understand the synchronization failures and lack of ovulation in FTAI protocols based on E2/P4 and these failures may be associated with diseases in dairy cows. Clinical diseases decrease the fertility in dairy cows (RIBEIRO et al., 2013), and in Brazil there were no studies associating disease with fertility, neither by estrus detection nor in cows subjected to FTAI protocols.

Supplementation with P4 is associated with greater embryo size (CLEMENTE et al., 2009). Nevertheless, it was observed that cows supplemented with P4 did not have increased fertility when subjected to AI. Moreover, when P4 supplementation was used in lactating recipient dairy cows a decreased fertility was observed. As discussed on chapter 2 and 3 of this present thesis, probably the benefit caused to increase conceptus is lost due to induced early luteolysis. This signals the need to study P4 supplementation associated with an antiluteolytic strategy. Thereby, it is likely that the beneficial effects of P4 supplementation on fertility of dairy cows can be obtained.

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