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Progesterone-based fixed-time artificial insemination protocols for dairy cows

Leonardo de França e Melo

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

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CONTENTS

RESUMO

Protocolos de inseminação artificial em tempo fixo à base de progesterona para vacas leiteiras

A produção de leite em bovinos aumentou consideravelmente nos últimos 50 anos. Inversamente, a eficiência reprodutiva vem diminuindo consistentemente. Vários fatores estão envolvidos, tais como manejo e ambiência, fatores fisiológicos e nutricionais, desafios sanitários, entre outros. As concentrações sanguíneas de hormônios esteroides em vacas leiteiras são baixas, devido à elevada ingestão de matéria seca e ao elevado fluxo sanguíneo hepático e metabolismo, os quais estão associados às alterações na expressão do estro e na qualidade de gametas, reduzindo assim a fertilidade. Entretanto, com o largo uso de programas de IATF, a fertilidade vem aumentado bem como a eficiência reprodutiva. Em função da grande quantidade e variações nos protocolos de IATF à base de E2/P4, três estudos foram realizados envolvendo diferentes combinações hormonais, os quais estão apresentados em dois capítulos desta tese. O primeiro estudo objetivou comparar a dinâmica ovariana e a fertilidade com o uso de dois tratamentos hormonais ao início do protocolo à base de P4, GnRH vs. BE, combinados com dois tratamentos no final do protocolo, BE vs. ECP. Para este estudo, 1.035 vacas lactantes foram aleatorizadas em um de quatro tratamentos: GnRH-BE, GnRH-ECP, BE-BE, BE-ECP. Interações e os tratamentos ao final do protocolo não afetaram a fertilidade. No entanto, o GnRH no início do protocolo tendeu a melhorar a P/IA, comparado ao BE, o qual foi responsável por uma grande proporção de vacas regredindo o CL durante o protocolo. O segundo estudo objetivou comparar as concentrações plasmáticas de P4 em vacas holandesas não lactantes entre dispositivos intravaginais de P4 novos (Novo), ou com 8 dias de uso, previamente autoclavados (Aut) ou desinfetados (Des) e contendo 1,9 ou 1,0 g de P4. Em um arranjo fatorial 2x3, aleatorizou-se 24 vacas em dois dos seis tratamentos (duas réplicas). A P4 circulante média nos 8 dias com implante de P4 foi a seguinte em relação aos tratamentos: 1,9 g > 1,0 g; 1,9 g: Aut > Novo > Des; 1,0 g: Aut = Novo > Des ($P < 0.05$). O terceiro experimento objetivou comparar as concentrações de P4, a dinâmica ovariana e a fertilidade durante o uso de implante Aut ou Des com 1,9 g de P4, em 349 vacas holandesas lactantes submetidas a um protocolo de IATF à base de E2/P4, combinado com GnRH no início do protocolo. Pequenas variações foram verificadas entre os tratamentos nas concentrações de P4, porém sem efeito na dinâmica folicular, na taxa de sincronização e na P/IA. Contudo, a ciclicidade ou a ovulação no início do protocolo influenciaram variáveis reprodutivas, tais como o momento e a sincronização da emergência da onda folicular, a proporção de vacas em cio ao final do protocolo e o tamanho do folículo ovulatório. Além disso, mais vacas sincronizadas ao protocolo ficaram gestantes.

Palavras-chave: Bovino; Hormônio; Sincronização; Implante; Fertilidade; *Bos taurus*

ABSTRACT

Progesterone-based fixed-time artificial insemination protocols for dairy cows

In the last 50 years, milk production increased in lactating dairy cows. In contrast, reproductive efficiency has dramatically decreased. Several causes may be involved, such as management and environmental factors, physiological and nutritional factors, disease challenges and others. Steroid hormone concentrations in high-producing lactating dairy cows are often at lower levels, due to high dry matter intake and increased liver blood flow and steroid hormones metabolism, which is associated with the compromised estrus expression and reduced oocyte quality, thus decreasing fertility. However, with the largely use of FTAI programs, fertility has turned the corner with current reports of increasing reproductive efficiency. Given the great number and variations on E2/P4-based FTAI protocols, three studies were performed involving different hormonal combinations and are presented in two chapters in this thesis. The first study aimed to compare the ovarian dynamics and fertility using two different treatments at the initiation of a P4-based FTAI protocol, GnRH vs. EB, combined with two different treatments at the end of the protocol, EB vs. ECP. For this study, 1,035 lactating cows were completely randomized into one of four treatments: GnRH-EB, GnRH-ECP, EB-EB, EB-ECP. Interactions and treatments at the end of the protocol did not affect fertility. However, GnRH rather than EB at the beginning tended to increase P/AI and greater proportion of cows regressed the CL when EB was used. The second study aimed to compare plasma P4 concentrations in non-lactating Holstein cows fitted implanted with new (New), or 8-days used intravaginal P4 implants previously autoclaved (Aut) or disinfected (Dis), and containing 1.9 or 1.0 g of P4. Using a 2x3 factorial arrangement of treatments, 24 cows were randomly assigned to two of six treatment groups (two replicates). Mean circulating P4 during 8 days with P4 implant were the following regarding treatments: 1.9 g > 1.0 g; 1.9 g: Aut > New > Dis; 1.0 g: Aut = New > Dis ($P < 0.05$). The third experiment was performed with 349 cows in two farms and aimed to compare P4 concentrations, ovarian dynamics and fertility during use of 1.9 g Aut or Dis intravaginal P4 implants, in lactating Holstein cows submitted to a 10-day long E2/P4-based FTAI protocol, combined with GnRH treatment at the beginning of the protocol. Slight variations in P4 concentrations were observed between treatments, which did not affect follicular dynamics, synchronization rate or P/AI. However, presence of CL or ovulation at the beginning of the FTAI protocol affected several reproductive variables, such as the time and synchronization of the follicular wave emergence, proportion of cows in estrus at the end of the protocol and size of the ovulatory follicle, and more overall synchronized cows became pregnant to the FTAI protocol.

Keywords: Bovine; Hormone; Synchronization; Implant; Fertility; *Bos taurus*

1 INTRODUCTION

The use of artificial insemination (AI) in commercial dairy herds continues to allow consistent improvements in the genetics of dairy cattle worldwide (VISHWANATH, 2003). However, the challenges of maintaining good reproductive performance in dairy herds that use AI have also been well-recognized, with reports of reduced detection of estrus (WASHBURN et al., 2002), elevated risks of anovulation (SARTORI; ROSA; WILTBANK, 2002; WILTBANK; GUMEN; SARTORI, 2002), and declining pregnancies per AI (P/AI; BUTLER, 2000; LUCY, 2001; WASHBURN et al., 2002). Multiple factors likely contribute to reduced reproductive efficiency in dairy herds, including: management and physiologic factors (CARAVIELLO et al., 2006; SCHEFERS et al., 2010), increasing metabolism of steroid hormones (WILTBANK et al., 2006), genetic factors (NORMAN et al., 2009), health challenges (CHEBEL et al., 2004; RIBEIRO et al., 2013), and nutritional factors (BUTLER, 2000; CARVALHO et al., 2014). However, the declining reproductive performance seems to have turned the corner with current reports of increasing reproductive efficiency (NORMAN et al., 2009), coinciding with the introduction of programs for precise synchronization of ovulation and fixed-time AI (FTAI; BISINOTTO et al., 2014; WILTBANK; PURSLEY, 2014).

Fixed-time AI protocols are largely used worldwide and represent an important reproductive management tool to improve reproductive efficiency and profitability of commercial dairy herds (NORMAN et al., 2009). These protocols have been developed and allow AI to be performed at a known time, in relation to expected ovulation without the need for detection of estrus (SANTOS et al., 2010; SOUZA et al., 2008; PEREIRA et al., 2014), even in anovular cows (BISINOTTO et al., 2010; STEVENSON et al., 2006). There have now been numerous modifications and improvements to the original protocols (BINELLI et al., 2014; PURSLEY; MEE; WILTBANK, 1995; WILTBANK; PURSLEY, 2014), but both present the same objectives and use a combination of GnRH, PGF, progesterone (P4)/progestin, and/or E2 esters (BARUSELLI et al., 2012; BINELLI et al., 2014). Despite the advantages of synchronizing the follicular wave emergence, controlling the corpus luteum (CL) function and synchronizing the ovulation at the end, several cows do not respond to protocol (MONTEIRO Jr. et al., 2015) and the development of an efficient FTAI protocol is of a great challenge for many researchers.

Progesterone is a steroid hormone primarily secreted by the CL and placenta, and circulating P4 concentration is determined by a balance between P4 production and P4 metabolism, primarily by the liver (WILTBANK et al., 2014). The importance of P4 for fertility and pregnancy maintenance is very well documented and decreased fertility in lactating dairy cows is often associated with decreased concentrations of circulating P4 (WILTBANK et al., 2006). Compared to non-lactating dairy cows and heifers, lactating cows have lower plasma concentrations of P4 (SARTORI et al., 2004) due to high hepatic metabolism of steroid hormones induced by the elevated dry matter/energy intake (WILTBANK et al., 2002). Beyond that, the importance of P4 concentrations during and after FTAI protocols is very well documented (BISINOTTO et al., 2015; CERRI et al., 2011a; PEREIRA et al., 2014) and supplementation of P4 during the follicular wave development by using intravaginal devices (BISINOTTO et al., 2015) may increase fertility specially in anovular (CERRI et al., 2009) or in high-producing dairy cows presenting decreased concentrations of P4, due to increased metabolism of steroid hormones (SARTORI; ROSA; WILTBANK, 2002; WILTBANK et al., 2006).

Intravaginal P4 devices have been used for decades with the aim of controlling the estrous cycle in farm animals (ZULUAGA; WILLIAMS, 2008) and other purposes (BISINOTTO et al., 2013; GUMEN; WILTBANK, 2005). There are several types of intravaginal P4 inserts commercially available worldwide and most of them comprises a T-shaped vaginal implant containing different amounts of P4 in silicone molded over a nylon spine, designed for slow hormonal delivery into the blood circulation and have been incorporated successfully in synchronization of ovulation protocols (GALVÃO et al., 2004; STEVENSON et al., 2006).

Although not recommended by the manufacturers, in many countries the reuse of intravaginal devices is a common method to reduce costs of the synchronization programs, as they still present residual P4 after a certain insertion period (CERRI et al., 2009). Various strategies have been used to process the inserts prior to reuse, such as disinfection or sterilization (CERRI et al., 2009; LONG et al., 2009; ZULUAGA; WILLIAMS, 2008). Moreover, sterilization, based on high pressure at elevated temperature during certain period of time, may modify the structure of the used inserts, leading to a greater release of P4 over an insertion period, compared to disinfected inserts (ZULUAGA; WILLIAMS, 2008), and provide a better endocrine environment during reuse in high-producing lactating dairy cows undergoing to FTAI protocols.

Therefore, experiments were conducted to evaluate the use of different hormones on an E2/P4-based FTAI protocol.

1.1 Hypotheses

1.1.1 Initiation of a FTAI protocol with GnRH compared to EB would increase fertility in lactating dairy cows, due to improved endocrine environment during ovulatory follicle growth, in spite of reduced follicular wave synchronization;
1.1.2 Induction of ovulation at the end of the protocol with EB would improve fertility compared to ECP, due to the expected more physiological pattern of circulating E2 during the proestrus period;
1.1.3 The effect of treatments at the beginning of the protocol and end of the protocol would be additive;
1.1.4 Plasma P4 concentrations during use of a new 1.9 g intravaginal P4 implant would be similar to the profile for a new 1.0 g intravaginal P4 implant;
1.1.5 Independent of method of disinfection, plasma P4 concentrations during treatment with a reused implant would be greater for a 1.9 g implant compared to a 1.0 g implant;
1.1.6 Independent of type of implant, plasma P4 concentrations would be greater for an autoclaved reused implant than for a chemically-disinfected reused implant;
1.1.7 The combination of GnRH and EB at the beginning of the protocol would produce better synchronization of the wave emergence in cows treated with autoclaved P4 implants, due to the rapid P4 increase after treatment with autoclaved inserts, compared to disinfected P4 inserts;
1.1.8 Elevated P4 concentrations during the FTAI protocol would produce better fertility in cows treated with an autoclaved insert compared to a chemically-disinfected insert.

1.2 Objectives

1.2.1 To compare the ovarian dynamics and fertility using two different treatments at the initiation of a FTAI protocol, GnRH vs. EB, combined with two different treatments at the end of the protocol, EB vs. ECP;
1.2.2 To compare plasma P4 concentrations in non-lactating Holstein cows during reuse of intravaginal P4 inserts (1.9 g or 1.0 g), that had been previously disinfected using an autoclave or by chemical disinfection;

1.2.3 To compare ovarian dynamics and fertility in lactating dairy cows that were treated with reused 1.9 g intravaginal P4 implants that were previously autoclaved or chemically disinfected as part of a 10-day long E2/P4-based FTAI protocol, combined with GnRH treatment at the beginning of the protocol.

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2 PROGESTERONE-BASED FIXED-TIME ARTIFICIAL INSEMINATION PROTOCOLS FOR DAIRY COWS: GNRH VS. ESTRADIOL BENZOATE AT INITIATION AND ESTRADIOL CYPIONATE VS. ESTRADIOL BENZOATE AT THE END

Abstract

The objectives were to evaluate ovarian dynamics and fertility comparing two treatments at the start of a progesterone (P4)-based fixed-time artificial insemination (FTAI) protocol, and two treatments at the end of the protocol. Thus, 1,035 lactating Holstein cows were assigned in a random phase of the estrous cycle to one of four treatments using a completely randomized design with a 2x2 factorial arrangement. At the beginning of the protocol (d-10), cows received GnRH or estradiol benzoate (EB) and at the end EB (d-1) or estradiol cypionate (ECP; d-2), resulting in four treatments: GnRH-EB, GnRH-ECP, EB-EB and EB-ECP. All cows received an intravaginal P4 device on d-10 which was removed on d-2. Cows also received PGF2α (PGF) on d-3 and d-2. The FTAI was performed on d0. Ovaries were evaluated by ultrasound for corpus luteum (CL) presence and regression (d-10 and d-3) and follicle measurements (d-10 and d0), as well as the uterus for percentage pregnant per AI (P/AI; d32 and d60). Blood samples were collected (d-10 and d-3) for P4 measurements. Treatment with GnRH rather than EB tended to increase P/AI on d32 (38.2 vs. 33.7%) and on d60 (32.9 vs. 28.9%). More cows treated with GnRH had CL on d-3 compared with EB-treated cows (77.3 vs. 58.3%), due to less CL regression between d-10 and d-3 (24.7 vs. 43.8%) and more cows with a new CL on d-3 (35.9 vs. 25.0%). Cows treated with GnRH also had greater P4 concentrations on d-3 than EB cows (3.4 vs. 2.0 ng/mL). Increased circulating P4 at the start of the protocol (d-10) decreased the probability of ovulation to EB or GnRH at that time. Cows from GnRH group also ovulated a larger diameter follicle at the end of the protocol (15.5 vs. 14.7 mm). No difference between EB and ECP in P/AI on d32 (34.8 vs. 37.0) and d60 (30.8 vs. 31.0%) or in pregnancy loss (11.1 vs. 15.4%) was detected and there was no interaction between treatments for P/AI. Independent of treatment, cows with CL on d-10 and d-3 had the greatest P/AI on d60 (36.9%). In conclusion, treatments at the end of the protocol were similar for ECP or EB and there was no additive effect or interactions on P/AI between treatments. However, cows treated with GnRH rather than EB on d-10 had less luteolysis and tended to have greater P/AI, probably because P4 concentrations were greater during the protocol. Finally, regardless of treatments, cows with CL at the beginning of the protocol as well as at the time of PGF had greater fertility.

Keywords: Bovine; Endocrine; Fertility; Synchronization

2.1 Introduction

In the United States, declining reproductive performance has been reported for more than 50 yr (WASHBURN et al., 2002), however, this trend seems to have been reversed in about 2000-2002 with current reports of increasing reproductive efficiency as measured by daughter pregnancy rate or days open (NORMAN et al., 2009). The improvement in reproductive

efficiency coincided with the introduction of programs for precise synchronization of ovulation and fixed-time AI (FTAI; WILTBANK; PURSLEY, 2014), although other changes during the last two decades, including improving genotype for fertility and management changes could also be important factors in the upsurge in reproduction on commercial dairy herds. Classical protocols to manage the estrous cycle produced a synchronized estrus by using PGF2α products or delivery systems for progesterone (P4) or progestin (BÓ et al., 2002; CAVALIERI et al., 2006). However, problems with detection of estrus and reductions in fertility were reported (LOPEZ et al., 2004). Protocols have now been developed that allow AI to be performed at a known time in relation to expected ovulation without the need for detection of estrus (SOUZA et al., 2008; PEREIRA et al., 2014), even in anovular cows (STEVENSON et al., 2006). There have now been numerous modifications and improvements to the original protocols (BINELLI et al., 2014; PURSLEY; MEE; WILTBANK, 1995; WILTBANK; PURSLEY, 2014). There are three main objectives for these protocols: 1) Synchronization of follicular waves to optimize the period of follicular dominance in order to not ovulate too old (CERRI et al., 2009) or too young (VASCONCELOS et al., 1999) of a follicle, 2) Synchronization of corpus luteum (CL) function and circulating P4, and 3) Synchronization of final ovulation with optimally scheduled FTAI. To achieve these objectives most protocols use a combination of GnRH, PGF, P4/progestin, and/or E2 esters (BINELLI et al., 2014) based on hormonal products approved for use in a specific country and attributes of different breeds, physiological conditions, and types of management.

Hormonal treatments at the initiation of a FTAI protocol are generally aimed at synchronizing the follicular wave by either ovulating a follicle using GnRH (PURSLEY; MEE; WILTBANK, 1995; VASCONCELOS et al., 2011) or suppressing current follicle growth using a combination of E2 and P4 (BÓ et al., 2002; BURKE et al., 1996; CAVALIERI et al., 2006; WILTBANK; PURSLEY, 2014). Treatment with GnRH at protocol initiation, as done in many countries, can induce an LH surge, ovulate a dominant follicle, and thus initiate a synchronized new follicular wave; although the magnitude of these responses depends on stage of the estrous cycle when GnRH treatment is given (GIORDANO et al., 2012b; RUTIGLIANO et al., 2008; VASCONCELOS et al., 1999). Treatment with the initial GnRH on Day 6 and 7 of the estrous cycle is the optimal time for ovulation and fertility, however, treatment with GnRH at a random reproductive stage generally results in ovulation in 50% or less of cows (BILBY et al., 2013; BISINOTTO et al., 2013; GIORDANO et al., 2012b; LOPES et al., 2013). In contrast to GnRH, E2/P4 treatments are used at the initiation of protocols in many countries and these protocols

seem to be effective at most, if not all, stages of the estrous cycle (MONTEIRO Jr. et al., 2015). Starting protocols with E2/P4 treatments suppresses circulating LH and FSH concentrations, and regresses the follicles in the current follicular wave (BÓ et al., 2002; BURKE et al., 1996; CAVALIERI et al., 2003; WILTBANK; PURSLEY, 2014), resulting in a synchronized follicular wave emergence 3 to 5 d later (BÓ et al., 1993) depending on the dose and type of E2 ester (BURKE et al., 2003). However, recent studies have reported that regression of the dominant follicle does not occur in all dairy cows following the standard treatment with 2 mg of E2-benzoate (EB) and an intravaginal P4 implant at the initiation of a protocol and that treatment with a larger dose of EB at protocol initiation (3 mg) does not improve follicle wave synchronization (MONTEIRO Jr. et al., 2015). Almost 30% of the cows did not initiate a new follicular wave and thus ovulated a larger persistent follicle at the end of the protocol resulting in much lower P/AI in these cows (MONTEIRO Jr. et al., 2015). Further, in contrast to treatment with GnRH that can cause ovulation of an accessory CL and thus increase circulating P4 during growth of the ovulatory follicular wave (SOUZA et al., 2008), treatment with E2-esters can induce CL regression, potentially decreasing circulating P4 during ovulatory follicle growth (MONTEIRO Jr. et al., 2015). Several studies have indicated a lower P/AI when the preovulatory follicle develops in the presence of lower circulating P4 (BISINOTTO et al., 2013; WILTBANK; PURSLEY, 2014). Thus, there may be advantages and disadvantages to protocols initiated with GnRH or E2/P4 in terms of synchronization of follicular waves, which seems to favor E2/P4, and hormonal environment during growth of the ovulatory follicular wave, which seems to favor GnRH.

At the end of FTAI protocols, ovulation is induced, generally by treatment with GnRH, EB, or E2-cypionate (ECP). The E2 esters are generally used at lower doses of EB or ECP at 0 (ECP) or 24 (EB) h after P4 implant removal, due to their pharmacodynamics, with these compounds producing a similar percentage of cows with synchronized ovulation but with differences in variability and timing of E2 peak, LH surge, and ovulation (SOUZA et al., 2009). Treatment with EB produced a circulating E2 peak at an earlier time, with greater peak E2, and of a shorter duration, compared to ECP (SOUZA et al., 2005), which should lead to a better synchronized ovulation. Nevertheless, the longer duration of circulating E2 after ECP than EB may produce a better endocrine environment during proestrus (BINELLI et al., 2014).

Thus, our overall objectives were to compare the ovarian dynamics and fertility using two different treatments at initiation of a FTAI protocol, GnRH vs. EB, combined with two different

treatments at the end of the protocol, EB vs. ECP. Thus, we had three specific hypotheses. 1) Initiation of a FTAI protocol with GnRH compared to EB would increase fertility in lactating dairy cows, due to improved endocrine environment during ovulatory follicle growth, in spite of reduced follicular wave synchronization. 2) Induction of ovulation at the end of the protocol with EB would improve fertility compared to ECP, due to the expected more physiological pattern of circulating E2 during the proestrus period. 3) The effect of treatments at the beginning of the protocol and end of the protocol would be additive. Thus, four FTAI protocols were compared for ovarian dynamics and hormonal concentrations during the protocol and fertility at the end of each protocol.

2.2 Materials and Methods

This experiment was conducted in a single commercial dairy farm, located in São Pedro city, São Paulo, Brazil. The Animal Use Ethics Committee of Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ) / University of São Paulo approved all procedures involving cows in this study.

2.2.1 Animals, housing and diets

For this study, a total of 1,035 lactating Holstein cows was used (363 primiparous and 672 multiparous). At the beginning of the experiment (d-10), cows averaged (mean ± SD) 140.7 ± 97.1 DIM, yielding 36.9 ± 9.33 kg of milk/d, with body condition score (BCS) of 2.8 ± 0.29 and lactation number of 2.2 ± 1.33. Cows were housed in free-stall barns with free access to water and mineral salt and fed *ad libitum* with a TMR diet based on corn silage and Tifton 85 hay as forages, and concentrate based on corn and soybean meal, minerals and vitamins balanced to meet or exceed the nutritional requirements of lactating dairy cows (NATIONAL RESEARCH COUNCIL - NRC, 2001). Throughout the experiment, cows were milked three times daily, 8 h apart.

2.2.2 Protocols and treatments

Cows were randomly assigned to one of four treatment groups using a completely randomized design with a 2x2 factorial arrangement of treatments. Cows were assigned to one of two treatments at the beginning of the FTAI protocol (d-10): EB (n = 485), where cows received 2.0 mg EB (Gonadiol, MSD Saúde Animal, São Paulo, Brazil); or GnRH (n = 550),

where cows received 100 µg Gonadorelin (Fertagyl, MSD Saúde Animal, São Paulo, Brazil). At the end of the protocol, cows were assigned to one of two treatments: ECP (n = 516), where cows received, on d-2, 1.0 mg of ECP (ECP, Zoetis, São Paulo, Brazil); or EB (n = 519), where cows received, on d-1, 1.0 mg EB. All cows received an intravaginal P4 implant containing 1.9 g of P4 (CIDR, Zoetis, São Paulo, Brazil) at initiation of the protocol (d-10). The implant was removed on d-2. Two treatments with 500 µg of cloprostenol sodium (PGF, Sincrosin, Vallée S.A., São Paulo, Brazil), on d-3 and d-2, were administered in all cows. Use of two PGF treatments in this study was based on results from previous studies showing that treatment with only a single PGF does not produce complete CL regression in all cows that were either treated with a GnRH protocol (WILTBANK et al., 2015) or an E2/P4 protocol (PEREIRA et al., 2013b; MONTEIRO Jr. et al., 2015). Cows were inseminated on d0 at 48 h after removal of the P4 implant using conventional frozen/thawed semen from Holstein sires (Figure 1).

2.2.3 BCS, DIM, Milk Yield and Ovarian Structures

At experiment enrollment, all cows were scored for body condition using a 1 to 5 point scale according to FERGUSON et al. (1994). For this study, BCS was categorized into low BCS (≤ 2.75) or moderate BCS (> 2.75) and also categorized by DIM into lower (≤ 120) or higher (> 120 d after calving). Based on parity, milk yield was categorized (above or below the average production within a parity) for primiparous cows (lower ≤ 33.0 kg/d and higher > 33.0 kg/d) and multiparous cows (lower ≤ 38.0 kg/d and higher > 38.0 kg/d) for further analyses.

From a subset of 418 cows, ovaries were evaluated using a transrectal ultrasound machine (Ibex Lite, E.I. Medical Imaging, Loveland, CO, USA) with an 8-5 MHz multi-frequency linear-array transducer on d-10, d-3 and d0 of the protocols. On d-10, ovaries were evaluated by ultrasound to confirm the presence or absence of a CL and to measure the diameter of the largest follicle. Ovaries were again evaluated by ultrasound on d-3, to confirm the presence or absence of CL and to determine whether CL regression occurred between d-10 and d-3 and whether ovulation occurred following the treatments on d-10. Ultrasound evaluation was also performed on d0 to measure the diameter of the ovulatory follicle. All measurements were done as described by SARTORI et al. (2004).

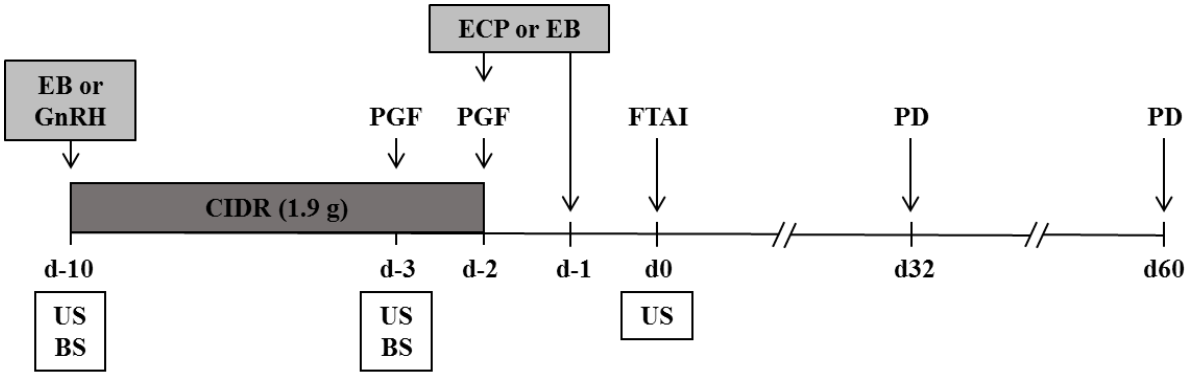

Figure 1 - Diagram of activities for the study. Study d-10 is the day the fixed time artificial insemination (FTAI) protocol began with cows receiving an intravaginal progesterone (P4; CIDR) implant and 2.0 mg estradiol benzoate (EB) or 100 µg gonadorelin (GnRH). On d-3 and d-2, 500 µg cloprostenol sodium (PGF) was administered. On d-2 the P4 implant was removed, and cows either received 1.0 mg estradiol cypionate (ECP) on the same day, or 1.0 mg EB on d-1. All cows were inseminated on d0. Pregnancy diagnosis (PD) was performed on d32 and confirmed on d60. The study was performed using a completely randomized design with a 2x2 factorial arrangement of treatments. US = ovary scanned by ultrasound; and BS = blood sample for circulating P4 concentration. On d 10, cows received fixed time AI (FTAI). Transrectal ultrasonography **(**US**)** was performed day from d 0 to ovulation (or d 12)

2.2.4 Blood Collection and Progesterone Assay

From a subset of 340 cows on d-10 and 376 on d-3, blood samples were collected by puncture of the coccygeal vein or artery into 10 mL evacuated tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA) for P4 measurements, immediately before administration of treatments on d-10 and on d-3. Immediately after collection, samples were placed in ice and transported to the laboratory within 5 h and kept refrigerated overnight. Blood tubes were centrifuged at 1900 g for 15 min at 4˚C and serum was frozen at -20˚C for further analyses of P4 by a solid-phase RIA using a commercial kit (Coat-A-Count; Siemens Healthcare Diagnostic, Los Angeles, CA, USA). The assay sensitivity was 0.01 ng/mL and the intra- and inter-assay CVs were 4.27% and 9.11%, respectively.

2.2.5 Pregnancy Diagnosis and Reenrollment of Previously Synchronized Cows

Pregnancy diagnosis was done at 32 d after AI by transrectal ultrasonography of the reproductive tract by confirming an embryo heartbeat. Pregnant cows were reconfirmed at 60 d after AI. Pregnancy per AI was calculated at d32 and d60 and pregnancy loss between these two evaluations. At any time during the experiment, cows that were diagnosed not pregnant were reenrolled in the experiment for further resynchronization.

2.2.6 Statistical Analysis

To test the hypotheses for the main treatment effects at the beginning of the protocol, a one-tailed test was used, based on our previous hypothesis that GnRH would be superior to EB at the beginning of a protocol. At the end of the protocol, we were not certain of which treatment would be better due to lack of previous results and therefore a two-tailed test was utilized. To test for interactions between treatments, two-tailed tests were used. The sample size was calculated using the Minitab statistical software (version 17.3.1; Minitab Inc., State College, PA) in order to detect statistical significance considering $\alpha = 0.05$ and $\beta = 0.20$. For binary data, 500 cows per treatment were deemed necessary to detect an increase of approximately 5 percentage units (e.g., 30.0 vs. 35.2%) only taking into account the main effect of hormonal treatment. For serum P4 concentration a standard deviation of 3.0 ng/mL was used in order to detect a difference of 0.7 ng/mL. For this, the number of cows needed per treatment was approximately 145.

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 response. The models included the fixed effects of treatment on d-10, treatment on d-2 (or d-1), parity as primiparous and multiparous, categorized milk yield within parity as below or above the mean value, categorized DIM as below or above 120 DIM, categorized BCS as low or moderate, season (summer and fall as hot, winter and spring as cold) the interactions between treatments at the beginning and at the end of the protocol, treatments and parity, treatments and categorized milk yield, treatments and categorized DIM, treatments and categorized BCS, and treatments and season. The estimates were back-transformed using the ILINK function of SAS to generate the adjusted Tukey percentages, and the results are expressed as Least Square Means ± Standard Error of Means (LSM ± SEM). 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 LOGISTIC procedure of SAS was used for logistic regression to model the probability of ovulation after GnRH and EB treatments as a function of P4 concentration at the beginning of the protocol, and the probability of pregnancy on d 60 after AI as a function of P4 concentration on d-10 and d-3.

The continuous data such as size of the largest follicle on d-10, size of the largest follicle on d0, and serum P4 concentrations on d-10 and d-3 were analyzed using the MIXED procedure

of SAS version 9.3. Data were tested for normality of residuals using the UNIVARIATE procedure of SAS. The P4 data were analyzed as nonparametric using the Kruskal-Wallis test ordered by the RANK procedure of SAS. The models included the fixed effects of treatment on d-10, treatment on d-2 (or d-1), parity, categorized milk, categorized DIM, categorized BCS, season, the interactions between treatments at the beginning and at the end of the protocol, treatments and parity, treatments and categorized milk yield, treatments and categorized DIM, treatments and categorized BCS, and treatments and season. The Kenward-Roger method was used to calculate the denominator degrees of freedom to approximate the F-tests in the mixed models. The estimates were back-transformed using the PDIFF function of SAS to generate the adjusted Tukey comparisons of means.

The results are expressed as least square means ± standard error of means (LSM ± SEM). For all analyses, only variables with $P < 0.20$ were kept in the final model, unless the variable was essential, such as treatments and their interactions. Differences were considered significant when $P \leq 0.05$, whereas a tendency was defined as $0.10 \geq P > 0.05$.

2.3 Results and Discussion

Our first hypothesis was that starting the FTAI protocol with GnRH compared to EB would increase fertility in lactating dairy cows, due to improved endocrine environment during ovulatory follicle growth, in spite of, potentially, reduced follicular wave synchronization. This idea was based on the findings of previous studies that 25-30% of cows did not properly synchronize follicular wave emergence when E2/P4-based programs were used (MONTEIRO Jr. et al., 2015) and also that EB can induce luteolysis (ARAUJO et al., 2009). This hypothesis was conditionally accepted based on the results that initiation of the protocol with GnRH rather than EB treatment tended to increase P/AI on d32 ($P = 0.07$) and on d60 ($P = 0.09$) after AI (Table 1). The absolute difference in P/AI was 4.5% (38.2 – 33.7) which translates into 13.4% more pregnancies from the protocol (4.5/33.7) based on d32 pregnancy diagnosis or 13.8% more pregnancies (4/28.9) based on the d60 diagnosis. There was no difference ($P = 0.38$) in pregnancy loss between EB and GnRH treatment (13.7 vs. 12.5%).

Table 1 - Results (LSM ± SE) from cows that had the follicle wave synchronized on d-10 of the fixed-time artificial insemination (FTAI) protocol with estradiol benzoate (EB) or with GnRH

	EB[1]	**GnRH[1]**	***P***
CL on d-10, % (n/n)	70.3 ± 3.4 (137/197)	73.0 ± 3.1 (158/220)	0.27
Serum P4 concentrations on d-10, ng/ml (n)	3.0 ± 0.2 (163)	3.2 ± 0.2 (172)	0.27
CL on d-3, % (n/n)	58.3 ± 3.6 (113/197)	77.3 ± 2.9 (168/220)	< 0.01
Serum P4 concentrations on d-3, ng/ml (n)	2.0 ± 0.1 (172)	3.4 ± 0.2 (200)	< 0.01
CL regression between d-10 and d-3[2], % (n/n)	43.8 ± 4.2 (60/137)	24.7 ± 3.4 (39/158)	< 0.01
Diameter of the largest follicle on d-10, mm (n)	12.8 ± 0.3 (197)	12.9 ± 0.4 (221)	0.39
Ovulation after d-10[3], % (n/n)	14.3 ± 2.5 (28/197)	27.3 ± 3.0 (60/220)	< 0.01
Ovulation on d-10[4], % (n/n)	17.1 ± 3.0 (28/164)	33.6 ± 4.5 (60/180)	< 0.01
Ovulatory follicle diameter, mm (n)	14.7 ± 0.4 (146)	15.5 ± 0.3 (166)	0.02
Pregnancy per AI (all cows)			
32 d, % (n/n)	33.7 ± 2.2 (161/485)	38.2 ± 2.2 (203/550)	0.07
60 d, % (n/n)	28.9 ± 2.1 (138/485)	32.9 ± 2.1 (173/549)	0.09
Pregnancy loss[5], % (n/n)	13.7 ± 2.7 (23/161)	12.5 ± 2.4 (29/202)	0.38

[1] Cows received on d-10 of FTAI protocol an intravaginal progesterone (P4) implant and either 2.0 mg EB or 100 µg gonadorelin (GnRH)
[2] Percentage of cows with CL on d-10, but without CL on d-3 of the FTAI protocol
[3] Percentage of cows with a new CL on d-3 of the FTAI protocol
[4] Percentage of cows with a follicle diameter ≥ 10.0 mm on d-10 and with a new CL on the same ovary on d-3 of the FTAI protocol
[5] Pregnancy loss between gestation d32 and d60

As shown in Table 1, at the start of the protocol (d-10) a total of 70.7% of cows had a CL, with no differences between treatment groups ($P = 0.27$). Although this is higher than might be expected for cows after a previous breeding, it is similar to the 73.9% that was previously reported at the time a Resynch procedure was initiated (BILBY et al., 2013). There were also no differences between groups at the initiation of the protocol in circulating P4 ($P = 0.27$) or size of the largest follicle of d-10 ($P = 0.39$) indicating that, even though the study began at an unknown day of the estrous cycle, cows were well randomized within treatment groups. However, 7 d after initiation of the protocol (d-3), a greater ($P < 0.01$) percentage of cows that

were treated with GnRH had CL (77.3%) compared to cows treated with EB (58.3%). This was related to two factors. First, a greater ($P < 0.01$) percentage of cows treated with EB had CL regression between d-10 and d-3 (43.8%) compared to cows treated with GnRH (24.7%). Second, a greater ($P < 0.01$) percentage of cows had ovulation (new CL detected on d-3) if they were treated with GnRH than EB. Consistent with these findings, circulating P4 concentrations on d-3 were also greater ($P < 0.01$) in cows treated with GnRH (3.4 ng/mL) than EB (2.0 ng/mL). Thus, cows treated with GnRH were more likely to have a CL at the time of PGF treatment (d-3) and had greater circulating P4 concentrations during preovulatory follicle development than cows treated with EB. Consistent with this result, a previous study (VASCONCELOS et al., 2011) compared initiation of a protocol with GnRH compared to EB and reported greater ($P < 0.01$) serum P4 at 7 d after protocol initiation for GnRH than EB (2.89 vs. 2.29 ng/mL). Similarly, a previous study (PEREIRA et al., 2013a) comparing an E2/P4 protocol to a GnRH-based protocol (5-d Cosynch) also reported greater ($P < 0.01$) circulating P4 concentrations for GnRH than EB (2.66 vs. 1.66 ng/mL).

The finding of increased CL regression in response to EB is consistent with a recent study that reported that ~55% of cows treated with EB at the beginning of an E2/P4 protocol had luteolysis during the first 7 d of the protocol (MONTEIRO Jr. et al., 2015). Older research also reported that treatment with E2-esters (such as E2-valerate) induced CL regression in cows that were treated at various stages of the estrous cycle, although these studies used higher doses of estradiol-esters than we used in our study (PRATT et al., 1991; RAJAMAHENDRAN; WALTON, 1990; WILTBANK et al., 1961). Moreover, when heifers were challenged with 1.0 mg of EB on d13 after ovulation, luteolysis was induced in 100% of heifers (ARAUJO et al., 2009). Thus, induction of premature luteolysis in some cows treated with EB can explain some of the differences in circulating P4 on d-3 of a protocol initiated with EB compared to GnRH.

The other factor that could increase circulating P4 during the protocol in GnRH-treated cows was that a greater percentage of cows ovulated in response to GnRH at the start of the protocol than following EB. The ovulation incidence (Table 1) could be expressed comparing all cows that were treated with GnRH vs. EB (35.9 vs. 25.0%; $P = 0.02$) or if only cows with a follicle $\geq$ 10 mm at the initiation of the protocol were considered (33.6 vs. 17.1%; $P < 0.01$). Of particular interest, as serum P4 concentrations increased, the probability of ovulation decreased ($P < 0.01$) for both EB and GnRH treatments (Figure 2). The decrease in ovulatory response as circulating P4 concentrations increased was expected and is likely due to the previously reported decrease in the magnitude of the GnRH-induced LH surge in response to

increasing circulating P4 concentrations (COLAZO et al., 2008; DIAS et al., 2010; GIORDANO et al., 2012a). It is possible that greater ovulation incidence may have occurred with a different GnRH analog, such as lecirelin or buserelin (PICARD-HAGEN et al., 2015), or with a greater dose of gonadorelin (GIORDANO et al., 2012a). It was also interesting that 17% of cows that were treated with EB also ovulated after initiation of the protocol, as demonstrated by a new CL that was present 7 d later (d-3 of the protocol). Treatment with a P4 implant in conjunction with EB at the initiation of the protocol is expected to prevent an E2-induced GnRH/LH surge, preventing subsequent ovulation (BÓ et al., 1993; SOUZA et al., 2009). It seems likely that some but not all cows were undergoing a natural ovulatory process due to the random day of the cycle at protocol initiation. Therefore, we speculate that, in spite of the presence of a P4 implant, some of the cows had a GnRH/LH surge induced by the EB treatment and, as expected, ovulation was much more likely to occur in the presence of low rather than high circulating P4 concentrations (Figure 2). This is consistent with another study that observed ovulation in response to E2 treatment, in cows in the late estrous cycle, even if the cows were treated simultaneously with P4 (KASTELIC et al., 2004).

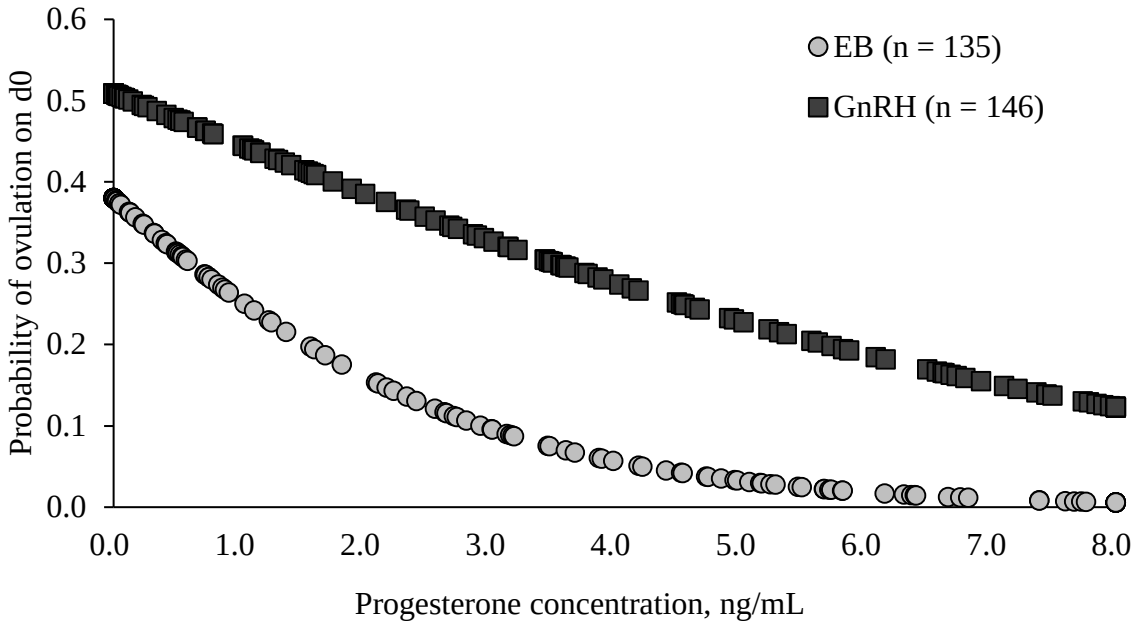

Figure 2 - Probability of ovulation after estradiol benzoate (EB) or GnRH administration in relation to serum progesterone concentrations at the time of initiation of the protocol. Only cows with follicles greater than 10.0 mm on d-10 were included in the analysis. There was a linear relationship for EB ($P < 0.01$) as well as for GnRH ($P < 0.01$) treatments

One other important difference between treatments at the beginning of the protocol was that cows that initiated the protocol with GnRH rather than EB ovulated a larger diameter follicle at the end of the protocol (15.5 vs. 14.7 mm; $P = 0.02$). It is well known that treatment with EB at the initiation of the protocol inhibits gonadotropin secretion and will initiate a new follicular wave 3 to 5 d later (BÓ et al., 1993; MONTEIRO Jr. et al., 2015; SARTORI et al., 2003; SOUZA et al., 2009). In a 10 d protocol, this would produce only 1 to 4 d of follicle dominance (3 to 5 d until wave emergence, 3 to 4 d from emergence to follicular deviation) at the time of AI. In contrast, cows in the GnRH group that ovulated to the GnRH, would have a new follicular wave during the first 1 to 2 d after treatment and follicular dominance of 3 to 6 d at time of AI. Cows in the GnRH group that did not ovulate to the GnRH could have substantial variability in degree of follicular dominance based on timing of follicular wave emergence. The larger ovulatory follicle is consistent with the idea that cows that initiated the protocol with GnRH had older follicles with a longer period of follicular dominance compared to cows treated with EB. Some studies have indicated that a greater period of follicular dominance is detrimental to follicles, leading to an aged oocyte at ovulation (CERRI et al., 2009; WILTBANK et al., 2011). However, juxtaposed to these changes, ovulation of a larger follicle will produce a larger CL (SARTORI et al., 2002) and greater circulating P4 after AI (VASCONCELOS et al., 2001), potentially leading to improved embryo elongation and development (CARTER et al., 2008; CLEMENTE et al., 2009). Furthermore, increased circulating P4 during preovulatory follicle development, as seen in this study in cows that received GnRH at the beginning of the protocol, has been associated with decreased LH pulse frequency which could help avoid overstimulation of the preovulatory follicle and associated oocyte (WILTBANK et al., 2011). Thus, increased circulating P4 during preovulatory follicle development, in spite of ovulating a larger and probably older follicle, may help explain the better fertility in cows that received GnRH at the initiation of the protocol, compared to those that received EB.

The second hypothesis of our study was that induction of ovulation with EB rather than ECP would produce better fertility to the timed-AI. This hypothesis was rejected based on the lack of difference between EB and ECP in P/AI at d32 ($P = 0.48$) and d60 ($P = 0.94$) or in pregnancy loss ($P = 0.22$; Table 2). In this study, the time of ovulation and the percentage of cows that ovulated after treatments was not evaluated. There are distinct differences in the patterns of circulating E2 after treatment with EB compared to ECP (SOUZA et al., 2005). Treatment with 1.0 mg EB compared to 1.0 mg ECP produced an earlier peak in circulating E2 (16.0 vs. 30.7 h after treatment) and a greater maximum E2 concentration (9.6 vs. 3.4 pg/mL),

although, ECP treatment produced a longer period with elevated E2 than EB (SOUZA et al., 2005). It was expected that EB treatment at the end of the protocol would produce greater synchrony in the time of ovulation, perhaps improving timing of ovulation and AI, but that ECP could provide a more optimal endocrine environment during proestrus due to a slower and less abrupt increase in circulating E2. Although EB and ECP are likely to have produced these differences in profiles of circulating E2, in the present study, there was no difference in size of the ovulatory follicle ($P = 0.90$; Table 2).

Table 2 - Results (LSM ± SE) for main effect of hormonal treatment at the end of the protocol. Cows had ovulation induced with estradiol cypionate (ECP), on d-2 of the fixed-time artificial insemination (FTAI) protocol, or with estradiol benzoate (EB), on d-1 of the protocol

	ECP[1]	**EB[1]**	***P***
Ovulatory follicle diameter, mm (n)	15.1 ± 0.3 (153)	15.1 ± 0.3 (186)	0.90
Pregnancy per AI (all cows)			
32 d, % (n/n)	37.0 ± 2.2 (186/516)	34.8 ± 2.2 (178/519)	0.48
60 d, % (n/n)	31.0 ± 2.1 (154/515)	30.8 ± 2.1 (157/519)	0.94
Pregnancy loss[2], % (n/n)	15.4 ± 2.8 (31/185)	11.1 ± 2.4 (21/178)	0.22

[1] Cows received 1.0 mg of ECP on d-2, or 1.0 mg of EB on d-1 of the FTAI protocol
[2] Pregnancy loss between gestation d32 and d60

Finally, our third hypothesis, that there would be an interaction or additive effects of the two treatments at the beginning or end of the protocol, was also rejected because there were no additive effects of treatments on P/AI at d32 or d60 or on pregnancy loss (Table 3). In addition, there was no interaction ($P > 0.10$) between treatments (EB-ECP, EB-EB, GnRH-ECP and GnRH-EB) for P/AI on d32 ($P = 0.83$) or d60 ($P = 0.52$), or pregnancy loss ($P = 0.38$). There was a consistent increase in P/AI (~4 to 5%) for cows that initiated the protocol with GnRH rather than EB, regardless of whether ovulation was induced at the end of the protocol with EB or ECP.

Table 3 - Fertility results (LSM ± SE) for all four treatment groups. Cows received estradiol benzoate (EB) or GnRH at the beginning of the fixed-time artificial insemination (FTAI) protocol, for wave synchronization, and estradiol cypionate (ECP) or EB at the end of the protocol, for ovulation synchronization

Wave synchronization	**EB[1]**		**GnRH[1]**	
Ovulation synchronization	**ECP[2]**	**EB[2]**	**ECP[2]**	**EB[2]**
Pregnancy per AI				
32 d, % (n/n)	35.1 ± 3.2 (80/233)	32.3 ± 3.0 (81/252)	39.0 ± 3.0 (106/283)	37.4 ± 3.0 (97/267)
60 d, % (n/n)	29.9 ± 3.0 (68/233)	27.9 ± 2.9 (70/252)	32.1 ± 2.9 (86/282)	33.8 ± 3.0 (87/267)
Pregnancy loss[3], % (n/n)	14.2 ± 3.9 (12/80)	13.1 ± 3.8 (11/81)	16.6 ± 3.6 (19/105)	9.4 ± 2.9 (10/97)

[1] Cows received on d-10 of the FTAI protocol an intravaginal progesterone (P4) implant and either 2.0 mg EB or 100 µg gonadorelin (GnRH) to synchronize the follicle wave
[2] Cows received 1.0 mg of ECP on d-2, or 1.0 mg of EB on d-1 of the FTAI protocol
[3] Pregnancy loss between gestation d32 and d60
P value > 0.10

Regardless of treatments, there was a quadratic effect of the circulating P4 concentrations either at d-10 ($P = 0.08$) or d-3 ($P = 0.05$) on P/AI, based on the d60 pregnancy diagnosis (Figure 3A). In addition, analysis of the presence of a CL at d-10 and/or d-3 compared to P/AI (Figure 3B), demonstrated a clear linear trend. Cows with no CL at the beginning and end of the protocol had the lowest P/AI (11.6%; 5/43), with cows with a CL present on only one of the days having intermediate values (21.2%; 36/170), and cows with CL at both d-10 and d-3 having the greatest P/AI (36.9%; 75/203). As described above, high concentrations of P4 during ovulatory follicle growth is important for oocyte quality (WILTBANK et al., 2006; 2014), which is associated with an increase in P/AI in dairy cows (BISINOTTO et al., 2013; WILTBANK; PURSLEY, 2014).

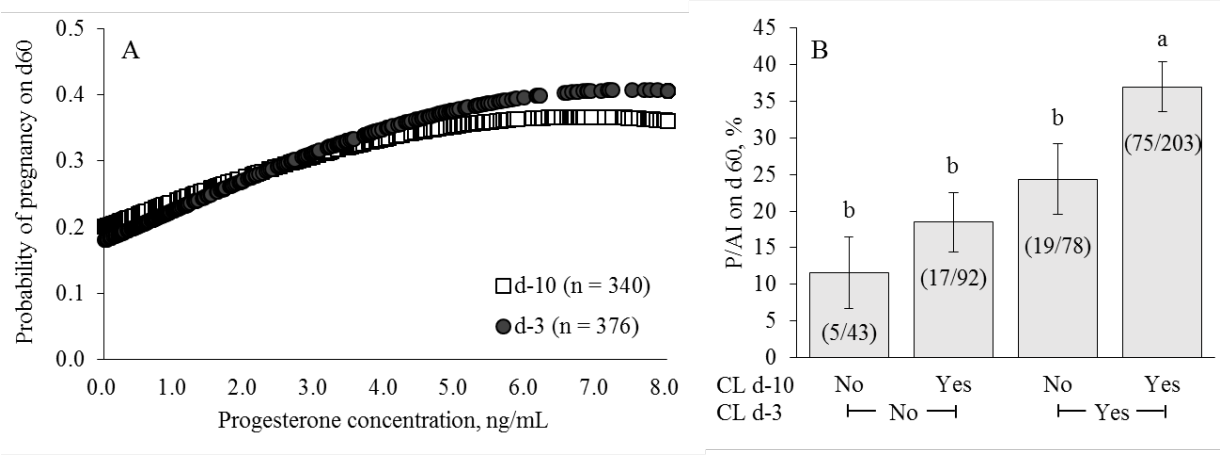

Figure 3 - A) Probability of pregnancy per AI (P/AI) compared to serum concentration of progesterone (P4), regardless of treatment, on d-10 (quadratic effect; $P = 0.08$) and d-3 (quadratic effect; $P = 0.05$) of a fixed-time artificial insemination (FTAI) protocol. B) P/AI, regardless of treatment, according to presence (YES) or absence (NO) of corpus luteum (CL) on d-10 and d-3 of the FTAI protocol. Different letters mean statistical differences ($P \leq 0.05$)

Finally, an analysis (Figure 4) was done of whether CL presence (YES) or absence (NO) on d-10 and d-3 were related to treatments that were given at the initiation of the protocol (GnRH vs. EB). The EB group (d-10) had greater ($P < 0.01$) proportion of cows with CL on d-10 and without CL on d-3 (YES-NO). In contrast, GnRH group had greater ($P < 0.01$) proportion of cows with CL on d-10 and d-3 (YES-YES) than EB group (Figure 4). The lack of difference between EB- and GnRH-treated cows for the NO-YES category may be explained by the low ovulatory response to GnRH and by the ovulatory response induced by EB (Figure 4). On the other hand, luteolysis induced by 2.0 mg of EB on d-10 may be responsible for more EB-treated cows in the YES-NO and fewer EB-treated cows in the NO-YES categories, compared to GnRH-treated cows. Thus, it seems likely that there is a change in the endocrine environment during preovulatory follicle development that is likely to favor fertility in the GnRH treatment group, due to the increase in percentage of cows that have a CL at the time of PGF treatment in the protocol.

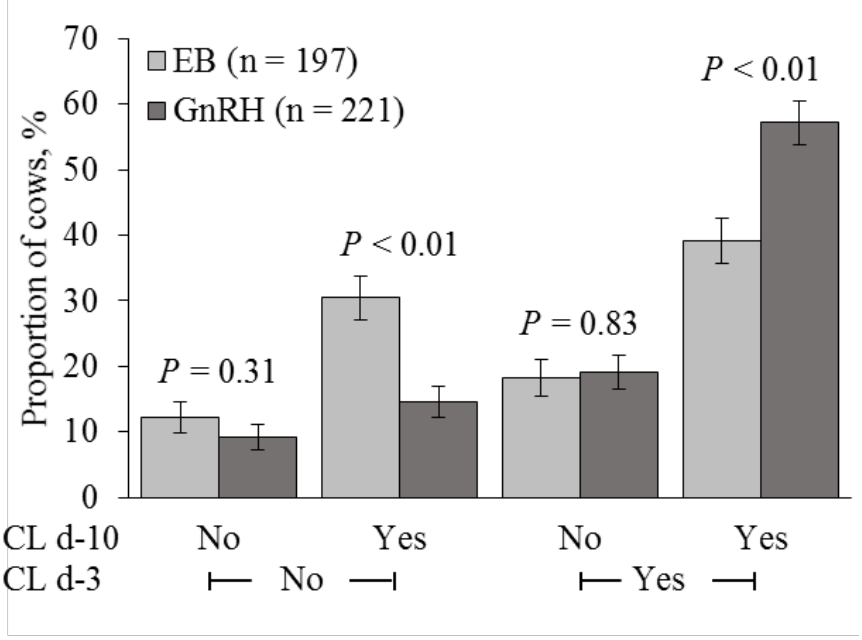

Figure 4 - Proportion of cows with (YES) or without (NO) corpus luteum (CL) on d-10 and d-3 of the fixed time artificial insemination (FTAI) protocol. *P* value indicates differences between the hormonal treatment groups (EB vs. GnRH) within the specific CL category (No-No, No-Yes, Yes-No, Yes-Yes)

Treatments at the beginning of the protocol affected (*P* = 0.02) ovulatory follicle diameter with cows treated with GnRH having larger ovulatory follicles than those treated with EB, as mentioned before (Table 1; Figure 5). However, treatments at the end of the protocol to synchronize ovulation (ECP vs. EB) did not affect (*P* = 0.89) the size of the ovulatory follicle (Table 2). Studies showed a quadratic effect for the probability of pregnancy according to ovulatory follicle diameter (PEREIRA et al., 2014; SARTORI et al., 2006; SOUZA et al., 2007). Another study described a linear effect between P/AI and ovulatory follicle size with greater P/AI with greater ovulatory follicle size in cows that had a CL present during the protocol (PEREIRA et al., 2015). In the present study, regardless of treatments, we observed that the P/AI on d60 after AI in cows with CL on d-10 and d-3 was not affected by the ovulatory follicle size (33.0% ± 4.6 vs. 34.9% ± 7.3 for follicle size 10-16 mm vs. > 16 mm, respectively; *P* = 0.83). However, cows without CL on d-10 and/or d-3 that ovulated follicles > 16 mm had lower (*P* = 0.05) P/AI on d60 (16.7% ± 4.4). Interestingly, 88.9% (16/18) of the cows that ovulated larger follicles (> 18.0 mm) and were treated with EB at the beginning of the protocol did not have CL on d-10 and/or d-3, compared to 58.3% (21/36) of the GnRH-treated cows (*P* < 0.01). Although these cows were treated with E2 and P4 on d-10 to induce emergence of a new follicle wave (BÓ et al., 1993), a previous study has demonstrated that atresia of the follicles did not happen in some of these cows and cows ovulated a persistent follicle

(MONTEIRO Jr. et al., 2015). In this case, the circulating P4 induced by the CIDR implant was not enough to completely block the E2-induced GnRH-LH release (ROBINSON et al., 2000) or EB + P4 did not efficiently block FSH release from the pituitary.

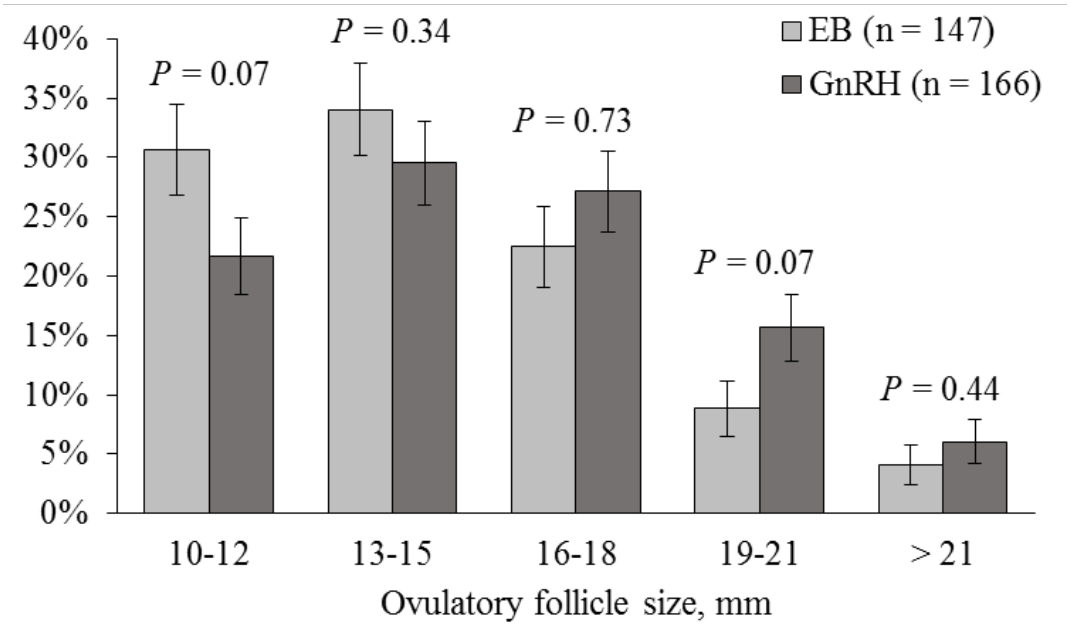

Figure 5 - Distribution of ovulatory follicle diameter on d0 of cows treated with estradiol benzoate (EB) or GnRH at the beginning of the protocol (d-10). An effect of treatment was observed (P = 0.02)

2.4 Conclusions

Cows that were treated with GnRH rather than EB at the initiation of a synchronization protocol tended to have better P/AI, probably due to increased circulating P4 concentrations during growth of the ovulatory follicle. The increased P4 was due to greater ovulation to the GnRH than the EB at the start of the protocol and reduced premature CL regression for GnRH compared to EB. However, treatments with either ECP or EB at the end of the protocol produced similar P/AI in this study. Additionally, there was no additive effect or interactions on P/AI between hormonal treatments at the beginning of the protocol (GnRH or EB) or hormonal treatments to synchronize ovulation at the end of the protocol (EB or ECP). Finally, independent of treatments, cows with CL at the initiation of the protocol as well as at the time of PGF had greater fertility.

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3 PLASMA PROGESTERONE CONCENTRATION IN NON-LACTATING HOLSTEIN COWS DURING REUSE OF INTRAVAGINAL PROGESTERONE DEVICES, PREVIOUSLY AUTOCLAVED OR DISINFECTED

Abstract

The aim of this study was to compare plasma progesterone (P4) concentrations in non-lactating, multiparous Holstein cows (n = 24) treated with two types of intravaginal devices containing 1.0 or 1.9 g of P4 either at the first use or during reuse of the implants after disinfecting the insert by autoclave or chemical disinfection. Cows were fed maintenance diet and water *ad libitum*. In a completely randomized design with a 2x3 factorial arrangement and two replicates, every cow underwent two of six treatments. Two sources of P4 [CIDR (1.9 g P4); Zoetis, and Sincrogest (1.0 g P4); Ourofino] and three types of processing: new (N), reused autoclaved (RA) and reused disinfected (RD) were used. After inducing luteolysis, to avoid endogenous circulating P4, the cows were randomized in one of six treatments (1.9 g N; 1.9 g RA; 1.9 g RD; 1.0 g N; 1.0 g RA and 1.0 g RD). Cows were treated with the implants for 8 days and during this period, blood samples were collected at the following times: 0, 2, 12, 24, 48, 72, 96, 120, 144, 168 and 192 hours. Statistical analyses were performed using Proc-Mixed and the averages and standard error (mean ± SE) of P4 concentrations were calculated using the Proc-Means procedures of SAS 9.4 ($P \leq 0.05$). No interaction between treatments was observed. Comparing types of implant, average P4 concentrations during treatments were greater for 1.9 g than 1.0 g (1.46 vs. 1.14 ± 0.04 ng/mL). When types of processing were compared, average P4 concentrations did not differ between autoclaved and new inserts (1.46 vs. 1.37 ± 0.05 ng/mL; respectively), but both were greater than disinfected implants (1.09 ± 0.04 ng/mL). Within 1.9 g P4 inserts, P4 concentrations from autoclaved implants were greater than new, which were greater than chemically disinfected (1.67 ± 0.06 vs. 1.49 ± 0.07 vs. 1.21 ± 0.05 ng/mL; respectively). For 1.0 g P4 implants, P4 concentrations from autoclaved did not differ from new, but both were greater than disinfected (1.20 ± 0.08 vs. 1.24 ± 0.06 vs. 0.97 ± 0.05 ng/mL; respectively). In conclusion, the mean plasma P4 concentration in non-lactating Holstein cows was greater for 1.9 g P4 than 1.0 g P4 and regardless of the type of implant, the autoclaving process provided greater circulating P4, in relation to disinfected, and similar or greater compared to the new.

Keywords: Hormone; Disinfection; Implant; *Bos taurus*

3.1 Introduction

Intravaginal progesterone (P4) inserts were developed to treat anovular cows but have also been used in whole herd synchronization programs (BISINOTTO et al., 2013; GUMEN; WILTBANK, 2005; ZULUAGA; WILLIAMS, 2008). There are several types of intravaginal P4 inserts commercially available worldwide with designs that allow retention within the vagina, usually with a T-shape, and prolonged delivery of P4, usually from P4-impregnated silicone molded over a nylon spine. In non-lactating ovariectomized cows, P4 inserts that have a similar surface area but contain 1.34 vs. 1.9 g of P4 release a similar amount of P4, on average,

620 and 610 mg of P4, respectively, over a period of 7 days (RATHBONE et al., 2002). These treatments produced circulating P4 of ~4 ng/mL on the day after insertion with concentrations at ~2.5 ng/mL by 7 days after insertion with no differences due to P4 load (10 to 30%; w/w; P4:silicone) or presence of additives (liquid paraffin, arachis oil, or polyethylene glycol), as long as surface area was kept constant (RATHBONE et al., 2002). However, increasing surface area of silicone available for release of P4 produced a linear increase in circulating P4, indicating the fundamental nature of this aspect of insert design. In anovular high-producing dairy cows, use of intravaginal P4 insert increased circulating P4 to only 0.8 to 1.0 ng/mL (CERRI et al., 2009; LIMA et al., 2009), probably due to the greater P4 metabolism related to elevated liver blood flow in lactating dairy cows (WILTBANK et al., 2006). Thus, surface area for release of P4 and physiology of treated cows seem to be major determinants of the circulating P4 concentrations produced by treatment with P4 inserts.

In many countries, the reuse of intravaginal inserts is a common method to reduce costs of synchronization programs, although not recommended by the manufacturers. For example, treatment of cows with a 1.9 g P4 insert for 7 days only removes ~600 mg of P4, leaving ~1.3 g of residual P4 load (RATHBONE et al., 2002). However, disinfection of the inserts prior to reuse is a major consideration, with producers primarily using either chemical disinfection of inserts or high-pressure steam sterilization using an autoclave (CERRI et al., 2009; LONG et al., 2009; ZULUAGA; WILLIAMS, 2008). Besides, the P4 profiles have not been extensively evaluated following these two methods of disinfection prior to reuse of different intravaginal P4 devices presenting different P4 loads.

Thus, the objective of this experiment was to compare plasma P4 concentrations in cyclic non-lactating Holstein cows during reuse of intravaginal P4 inserts (1.9 g or 1.0 g), that had been previously disinfected using an autoclave or by chemical disinfection. The hypotheses for this experiment were that: 1) Plasma P4 concentrations during use of a new 1.9 g intravaginal P4 implant would be similar to the profile for a new 1.0 g intravaginal P4 implant; 2) Independent of method of disinfection, plasma P4 concentrations during treatment with a reused implant would be greater for a 1.9 g implant compared to a 1.0 g implant; and 3) Independent of type of implant, plasma P4 concentrations would be greater for an autoclaved reused implant than for a chemically-disinfected reused implant.

3.2 Materials and Methods

This experiment was conducted at the Department of Animal Science facilities at Escola Superior de Agricultura “Luiz de Queiroz” (ESALQ)/University of São Paulo, located in Piracicaba city, São Paulo, Brazil. The Animal Research Ethics Committee of Escola Superior de Agricultura “Luiz de Queiroz” (ESALQ)/University of São Paulo approved all procedures involving cows in this study.

3.2.1 Cows, housing and diets

For this study, 24 non-lactating multiparous cycling Holstein cows were used. At the beginning of the experiment, cows averaged 600 kg of body weight (BW) and body condition score (BCS) of 3 (FERGUSON et al., 1994). Cows were kept in confinement with free access to water and mineral salt, and were fed a total mixed ration (TMR) maintenance diet (NRC, 2001) based on sugar cane bagasse as forage and concentrate based on corn and soybean meal, minerals and vitamins.

3.2.2 Protocols and treatments

Cows were randomly assigned to one of six treatment groups using a completely randomized design with a 2x3 factorial arrangement of treatments and two replicates, and every cow underwent two treatments. For this study, two sources of intravaginal P4 implants [CIDR (1.9 g); Zoetis, São Paulo, Brazil, and Sincrogest (1.0 g); Ourofino, Cravinhos, Brazil] and three types of processing [new (N), reused autoclaved (RA) and reused disinfected (RD)] were used, resulting in the following treatments: 1.9 g N, 1.9 g RA, 1.9 g RD, 1.0 g N, 1.0 g RA and 1.0 g RD.

At the beginning of the experiment (Day 0), all cows had their estrous cycle synchronized with a new 1.9 g P4 implant, that remained for 8 days. At 7 and 8 days after implant insertion, 25 mg of dinoprost tromethamine (PGF; Lutalyse, Zoetis) was administered and on Day 8, after the withdrawal of the P4 implant, a Norgestomet (Crestar; MSD, São Paulo, Brazil) ear implant was inserted, which was maintained for 48 hours to avoid ovulation and allow for a complete drop in circulating P4. On Day 10, cows were randomized in one of six treatments. The implants

were kept for 8 days and during this period, blood samples were collected for circulating P4 measurements at the following times: 0, 2, 12, 24, 48, 72, 96, 120, 144, 168 and 192 hours. On the last day, P4 implants were removed and Norgestomet was inserted again, and maintained for 48 hours, together with other PGF treatments at insertion and withdrawal times. Then, another replicate began on Day 20 similarly to the first replicate (Fig. 1).

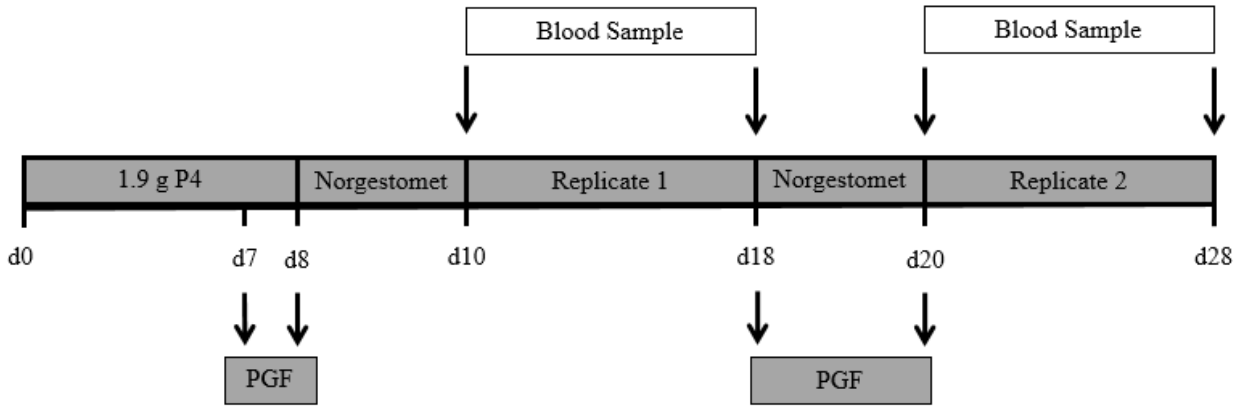

Figure 1 - Diagram of activities for Experiment 1 that compared plasma progesterone (P4) concentrations using a completely randomized block design with a 2x3 factorial arrangement of treatments and two replicates, with two intravaginal P4 devices (1.9 g and 1.0 g) and three types of processing [new (N), 8-d used autoclaved (RA) and 8-d used disinfected (RD)]. Day 0 is the beginning of the presynchronization protocol with cows receiving a new 1.9 g P4 device, that remained for 8 days, followed by two treatments of 25.0 mg of dinoprost tromethamine (PGF) on d7 and d8. After P4 insert removal, a Norgestomet ear implant was inserted for 2 days. After Norgestomet implant removal on d10, the first replicate began and cows were randomly assigned to receive one of six treatments (1.9 g N, 1.9 g RA, 1.9 g RD, 1.0 g N, 1.0 g RA, or 1.0 g RD). During the replicate, blood samples were collected on times 0, 2, 12, 24, 48, 72, 96, 120, 144, 168 and 192 hours for plasma P4 concentrations. At the end of the replicate on d18, devices were removed and another Norgestomet ear implant was inserted, remaining for 2 days and followed by a PGF treatment. On d20, after Norgestomet removal, another PGF was injected and the second replicate began, similar to the first, while cows were enrolled in a different treatment

3.2.3 Intravaginal P4 implants preparation

The autoclaved and disinfected implants were previously used in lactating dairy cows for 8 days. After removal, the inserts were washed in clean running water, and air dried at room temperature. Prior to use in the experiment, the inserts were autoclaved or disinfected. The protocol used to autoclave the P4 implants was similar to the one described by CERRI et al. (2009). Briefly, the inserts were placed in autoclave bags and autoclaved for 15 minutes at 121ºC and 725 mmHg. For disinfection, the implants were dipped for 15 minutes in 1:2000 diluted quaternary ammonia (CB-30 TA; Ourofino, São Paulo, Brazil) and air dried at room temperature as well.

3.2.4 Blood collection and P4 assay

Blood samples were collected by puncture of the jugular vein into 10 mL heparinized evacuated tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA) for plasma P4 measurements at the following times: 0, 2, 12, 24, 48, 72, 96, 120, 144, 168 and 192 hours. Blood samples at 0 hour were collected immediately before administration of treatments in both replicates on Day 10 and on Day 20, respectively, and at 192 hour immediately before implant withdrawal. After collection, samples were placed in ice and transported to the laboratory within 2 hours. Blood tubes were centrifuged at 1900 x g for 15 minutes at 4ºC and plasma was frozen at -20ºC. Plasma was analyzed for P4 by a solid-phase radioimmunoassay using a commercial kit (Coat-A-Count; Siemens Healthcare Diagnostic, Los Angeles, CA, USA). A single assay was performed with all samples. The assay sensitivity was 0.01 ng/mL and the intra-assay coefficient of variation was 4.6%.

3.2.5 Statistical Analysis

Data were tested for homogeneity of variances and normality of residuals using the GLM procedure of SAS version 9.4 (SAS/STAT, SAS Institute Inc., Cary, NC). Homogeneity of variances followed Hovtest and Welsh methods and normality of residuals were analyzed using the UNIVARIATE procedure of SAS, following Shapiro-Wilk method.

Concentrations of P4 were analyzed as repeated measures, using the MIXED Procedure of SAS. The replicate was considered a random effect and cow within time for the subject effect. The fixed effects of treatments, such as the type of implant, type of processing, time and their specific interactions with treatments were included in the model, fitting a Kenward-Roger method to calculate the denominator degrees of freedom to approximate the F-tests in the mixed models.

The estimates were calculated to generate the P-values from the adjusted Tukey comparisons of means, although the results are expressed as mean ± standard error of means (mean ± SE). Differences were considered significant when $P \leq 0.05$, whereas a tendency was defined as $0.10 \geq P > 0.05$.

3.3 Results and Discussion

Although no interaction was detected between type of implant and type of preparation method (P = 0.19), clear differences were found between the types of implant (1.0 vs. 1.9 g P4; P = 0.0002), implant preparation (Aut vs. Dis; P < 0.0001), and time (P < 0.0001) on circulating P4 concentrations. In addition, interactions were detected between types of implant and time (P = 0.05) and implant preparation method and time (P = 0.0002) on P4 concentrations. Mean P4 concentration was greater for the 1.9 g P4 than 1.0 g P4 implant, and lower for the disinfected than the autoclaved implant, during the 8 days of treatments (Table 1).

Table 1 - Plasma progesterone (P4) concentrations (mean ± SE) between 24 and 192 hours during the 8 days of treatments in 24 non-lactating dairy cows after insertion of intravaginal P4 implants (1.9 g or 1.0 g) that were submitted to three types of processing [new (N), autoclaved (A), or disinfected (D)], in a 2x3 factorial arrangement of treatments and two replicates. Every cow underwent two treatments

P4 insert	1.9 g	1.0 g	*P*-value	Average
New	1.49 ± 0.07^{b}	1.24 ± 0.06^{a}	0.04	1.37 ± 0.05^{a}
Aut	1.67 ± 0.06^{a}	1.20 ± 0.08^{a}	< 0.01	1.46 ± 0.05^{a}
Dis	1.21 ± 0.05^{c}	0.97 ± 0.05^{b}	0.02	1.09 ± 0.04^{b}
P value	< 0.05	< 0.01	-	< 0.01
Average	1.46 ± 0.04	1.14 ± 0.04	< 0.01	-

^{a,b}Values in the same column differ (P ≤ 0.05)

Our first hypothesis was that plasma P4 concentrations during the use of two new intravaginal implants containing 1.9 g and 1.0 g of P4 would be similar. This idea was based on the concept that when new inserts with the same surface area are used, even with different P4 loads, they are bioequivalent, having the same overall daily release of P4 during the first week of treatment (~0.61 g; RATHBONE et al., 2002). This hypothesis was rejected because the 1.9 g P4 implant had 28.1% greater (P = 0.04) circulating P4 concentrations compared to the 1.0 g P4 implant. This is much less than the 90% greater quantity of P4 load that is present in the 1.9 vs. 1.0 g P4 implants. This highlights the importance of surface area in determining circulating P4, although, P4 load has a minor but significant effect. Nevertheless, the repeated measures analysis (Fig. 2A) did not detect differences between specific days during the 8 days

of treatment with new 1.9 vs. 1.0 g P4 implants. In addition, there was no difference between 1.9 vs. 1.0 g P4 implants during the first 4 days (combined analysis) or the last 4 days of treatment, although there was a decrease in P4 during the last 4 days compared to first 4 days of treatment, irrespective of P4 load in implant ($P < 0.0001$; Fig. 4). Our results contrast, somewhat, with other results that show that P4 implants with different P4 load but similar surface area produced similar circulating P4 concentrations during the first week of implant treatment (RATHBONE et al., 2002). In contrast, residual P4 left in the implant after the first 7 days of treatment has been shown to be distinctly related to the initial amount of P4 in the new implant. For example, MacMillan and Peterson (1993) showed a quadratic relationship between the initial and residual amount of P4 over an insertion period of 15 days ($R^2 = 0.953$). An implant containing 0.69 g of P4 was almost completely depleted of P4 (0.07 g residual P4), whereas, implants with 1.25, 1.86, and 2.67 g of P4 lost ~1.0 g of P4 during the insertion period leaving dramatically different residual P4 in the used implants (0.31, 0.80, and 1.39 g P4, respectively). In addition, cows with widely varying feed intake and physiology had similar P4 release from a 1.9 g P4 implant during 11 days and similar residual P4 left in the implant after use for 11 days (RABIEE et al., 2001a, 2001b). In our experiment, we did not evaluate the residual P4 but would expect ~0.4 g of P4 to be released during the first 4-day period from either 1.0 or 1.9 g P4 implants and 0.25 to 0.3 g of P4 released during the next 4 days, based on previous results (RABIEE et al. 2001a, 2001b, 2002; RATHBONE et al., 2002). Thus, our first hypothesis was rejected due to small, but significant differences in P4 profiles during use of new P4 implants with differing initial P4 loads. In addition, the residual P4 amounts would be expected to be substantially different for 1.9 g (~1.2 g P4) compared to 1.0 g (~ 0.4 g P4) P4 implants when the implants were going to be reused for the second time in this experiment.

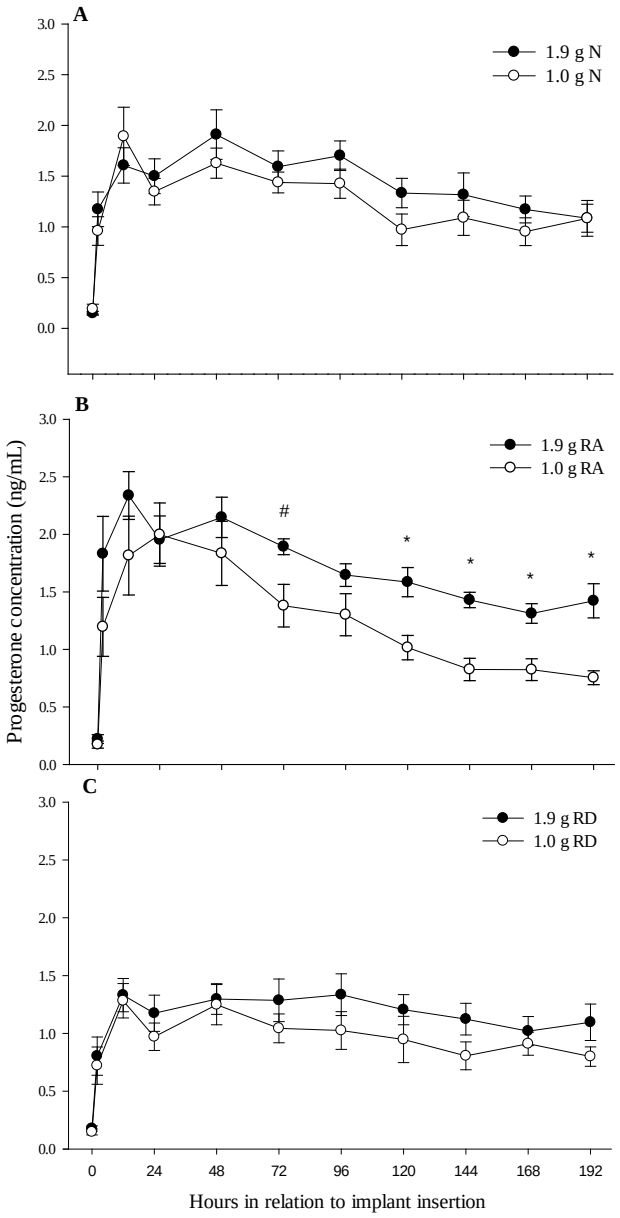

Figure 2 - Plasma progesterone (P4) concentrations during the 8 days of treatments in 24 non-lactating dairy cows using intravaginal P4 implants (1.9 g or 1.0 g) that were submitted to three types of processing [new (N), reused autoclaved (RA), or reused disinfected (RD)], in a 2x3 factorial arrangement of treatments and two replicates. Every cow underwent two treatments. Effect of treatment (P = 0.19); effect of implant (P = 0.0002); effect of processing (P < 0.0001); effect of time (P < 0.0001); interaction between treatment and time (P = 0.91); interaction between implant and time (P = 0.05) and interaction between processing and time (P = 0.0002). **Fig. A** shows plasma P4 concentrations from new intravaginal implants containing 1.9 g vs. 1.0 g of P4. **Fig. B** shows plasma P4 concentrations from 8-days used autoclaved implants containing 1.9 g vs. 1.0 g of P4. # Time 72 hours tended to differ (P = 0.08), and * times from 120 to 192 hours differed (P < 0.01). **Fig. C** shows plasma P4 concentrations from 8-days used disinfected intravaginal implants containing 1.9 g vs. 1.0 g of P4

Thus, our second hypothesis for this experiment was that, irrespective of disinfection method, the reused implant from the initial 1.9 g P4 implant would produce greater circulating P4 than the reused 1.0 g implant. This was based on the assumption that much greater residual

P4 would be available for release during reuse of the 1.9 than the 1.0 g P4 implant. This hypothesis was fully supported by the data for the reused implants. Mean P4 concentrations during the full 8-day period were 39.2% greater ($P < 0.01$) for autoclaved 1.9 vs. 1.0 g P4 implants and 24.7% greater for chemically-disinfected 1.9 vs. 1.0 g P4 implants (Table 1). This difference between types of implant was most apparent in cows treated with the autoclaved implants (Fig. 2B) with circulating P4 much greater for the 1.9 compared to the 1.0 g reused P4 implants. This difference was not detected using the repeated measures analysis of daily evaluations in cows treated with chemically-disinfected implants (Fig. 2C). It appears that autoclaving the implant caused more of the residual P4 to be "releasable" during the reuse period. However, this created a situation in which the residual P4 seemed to approach depletion from the autoclaved 1.0 g implant but not from the autoclaved 1.9 g implant (Fig. 2B). In the reused implants that were chemically disinfected, there was a lower quantity of residual P4 that was initially depleted for both types of implants and exhaustion of the residual P4 seemed to not be a problem by the end of the 8-day period, as circulating P4 was fairly constant (Fig. 2C). Previous studies have described the P4 profiles using implants with differing P4 loads but similar surface area in bilaterally ovariectomized non-lactating cows (RATHBONE et al., 2002) or when new or used intravaginal P4 implants were used in bilaterally ovariectomized non-lactating cows (ZULUAGA; WILLIAMS, 2008), or in high-producing dairy cows (CERRI et al., 2009). However, this is the first comparison of new and reused implants with differing P4 loads that were prepared by autoclaving as well as chemical disinfection. The autoclaving process may modify the structure of the implant or the location or disposition of P4 within the insert (ZULUAGA; WILLIAMS, 2008). The increased elution of P4 caused by the autoclaving process may produce more rapid subsequent depletion of the remaining P4, leading to exhaustion of P4 in the 1.0 but not the 1.9 g P4 implants. Nevertheless, in both previously used autoclaved implants, average P4 concentrations were generally greater than 1 ng/mL during the 8 days of treatment, which should be sufficient to prevent a GnRH-LH surge and properly synchronize the emergence of a new follicular wave in a P4-based FTAI protocol (BARUSELLI et al., 2012; WILTBANK et al., 2014).

Our third hypothesis was that circulating P4 would be greater for reused implants that were sterilized using an autoclave, rather than chemical disinfection, based on previous reports with autoclaved P4 implants (ZULUAGA; WILLIAMS, 2008). This hypothesis was conditionally accepted. The complete profiles for 1.9 (Fig. 3A) or 1.0 (Fig. 3B) g P4 implants demonstrate the effectiveness of autoclaving in causing residual P4 release. The autoclaved 1.9 g P4 implant

produced 38.0% greater circulating P4 than a disinfected 1.9 g P4 implant and even produced greater P4 concentrations (12.1%) than a new implant (Table 1). Similarly, the autoclaved 1.0 g P4 implant produced 23.7% greater circulating P4 than a disinfected 1.0 g P4 implant, although there was no difference between new and autoclaved 1.0 g P4 implants (Table 1). Figure 4 demonstrates the differences in circulating P4 during the first 4 days (24 to 96 hours) compared to the last 4 days (120 to 192 hours) of treatment with reused 1.9 vs. 1.0 g P4 implants that were previously autoclaved or chemically disinfected. The autoclaved reused 1.9 g P4 implant produced the greatest P4 concentrations during the first 4 days of treatment (1.83 ng/mL, on average) and this decreased 21.3% during the last 4 days of treatment (1.44 ng/mL). In contrast, cows treated with the chemically-disinfected 1.9 g P4 implant had no significant decrease in circulating P4 from the first 4 days vs. the last 4 days of treatment but values were significantly lower in both periods for cows treated with a chemically-disinfected vs. autoclaved 1.9 g P4 implant (Fig. 4). For the 1.0 g P4 implant, there was a dramatic decrease in circulating P4 during the first 4 days vs. the last 4 days of treatment with an autoclaved implant (40.4%) but also a smaller but significant decrease in circulating P4 between the first 4 days and the last 4 days in cows treated with the disinfected 1.0 g implant (20.2%). In summary, autoclaving compared to chemically-disinfecting increased circulating P4 during both the first 4 days (32.2%) and the last 4 days (22.2%) of treatment with a 1.9 g P4 implant, but only during the first 4 days (26.2%) and not during the last 4 days of treatment with the 1.0 g P4 implant (Fig. 4). Thus, solely based on P4 profile, reuse of P4 implants seems suitable when sufficient residual P4 remains in the implant and the releasable P4 is optimized by autoclaving the implant prior to reuse.

One other important consideration is that for new and autoclaved P4 implants there is a consistent decrease in circulating P4 concentrations over time after implant insertion ($P < 0.0001$), as previously reported (CERRI et al., 2009; MACMILLAN; PETERSON, 1993). It seems likely that most of the differences in P4 profiles observed in this study were related to alterations in P4 release from the implant since the vaginal mucosa has high permeability to steroid hormones and P4 subsequently enters the capillaries and blood stream by passive diffusion (ROTHEN-WEINHOLD et al., 2000). Indeed, P4 release from a silicone implant seems to follow a zero-order ($R^2 = 0.989$) release mechanism with particulate P4 in a saturating concentration at the interface of surfaces (RATHBONE et al., 2002). In this regard, treatment with 1, 2, or 3 P4 implants produced corresponding increases in circulating P4 (2X or 3X) with similar depletion of P4 from each of the implants, regardless of the number of implants that are

present in the vagina or the circulating P4 concentration (MACMILLAN; PETERSON, 1993). Nevertheless, differences in animal size (CERRI et al., 2009) and metabolic clearance rate for P4 (SANGSRITAVONG et al., 2002) can potentially alter the circulating P4 that is achieved in different cows or different experimental situations.

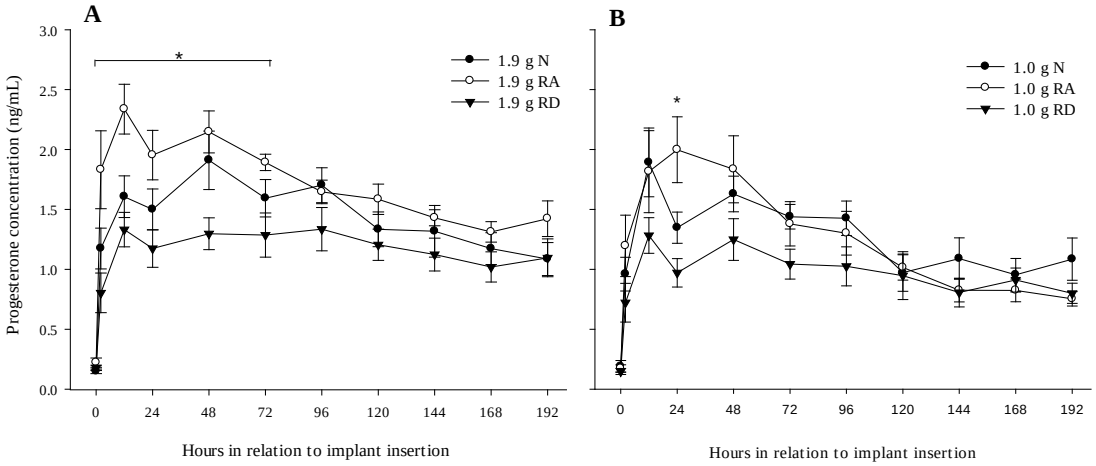

Figure 3 - Plasma progesterone (P4) concentrations during the 8 days of treatments with new (N), reused autoclaved (RA) or reused disinfected (RD) implants in 24 non-lactating dairy cows. Effect of treatment (P = 0.19); effect of implant (P = 0.0002); effect of processing (P < 0.0001); effect of time (P < 0.0001); interaction between treatment and time (P = 0.91); interaction between implant and time (P = 0.05) and interaction between processing and time (P = 0.0002). **Fig. A** is related to plasma P4 concentrations from 1.9 g intravaginal implants. *Time points from 2 to 72 hours are different (P < 0.05) between RA and RD. **Fig. B** is related to plasma P4 concentrations from 1.0 g intravaginal implants. *Time 24 hours is different (P < 0.0001) between RA and RD

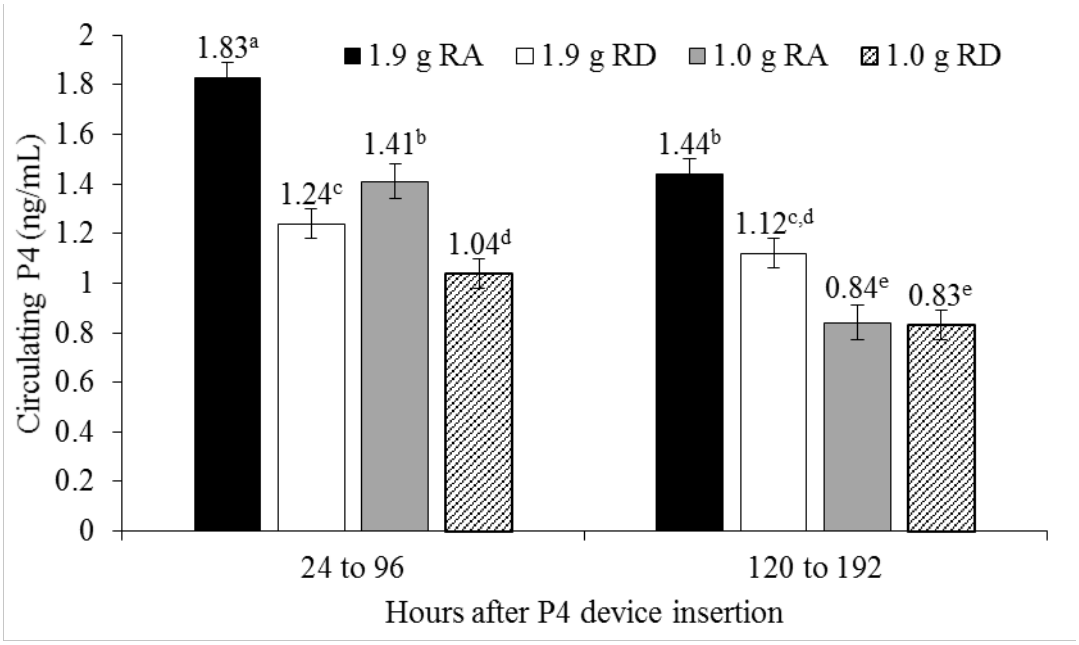

Figure 4 - Plasma progesterone (P4) concentrations (mean ± SEM) during the 8 days of treatments with 1.9 g reused autoclaved (RA; n = 8), 1.9 g reused disinfected (RD; n = 8), 1.0 g RA (n = 8), and 1.0 g RD (n = 8). Comparisons were done for the first 4 days and last 4 days after implant insertion. Effect of implant ($P < 0.001$); effect of processing ($P < 0.001$); effect of time ($P < 0.0001$); interaction between processing and time ($P < 0.001$) and interaction between implant, processing, and time ($P = 0.01$)
[a,b,c,d,e]Values differ ($P \leq 0.05$)

3.4 Conclusions

The mean plasma P4 concentration in non-lactating Holstein cows was greater for 1.9 g P4 than 1.0 g P4 and regardless of the type of implant, the autoclaving process provided greater circulating P4, in relation to disinfected, and similar or greater compared to the new.

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4 CIRCULATING PROGESTERONE, FOLLICULAR DYNAMICS, AND FERTILITY IN HOLSTEIN COWS DURING REUSE OF INTRAVAGINAL PROGESTERONE DEVICES FOR FIXED-TIME AI, PREVIOUSLY AUTOCLAVED OR DISINFECTED

Abstract

The objectives of this experiment were to compare circulating progesterone (P4), follicular dynamics, and fertility during reuse of P4 inserts for 8 days, that were previously autoclaved or chemically disinfected in lactating Holstein cows submitted to an estradiol (E2)/P4-based fixed-time artificial insemination (FTAI) protocol, combined with GnRH at the beginning of the protocol. For this study, 349 cows were used (123 primiparous and 226 multiparous) on two farms, averaging at the beginning of the protocol (mean ± SD) 163.9 ± 141.9 days in milk; 35.7 ± 11.3 kg/milk and body condition score of 2.9 ± 0.5. Cows were randomly assigned to one of two treatment groups using a completely randomized design of treatments. On d-10 (before AI), cows received reused implants (1.9 g CIDR; previously used for 8 d) that were disinfected either using autoclave (Aut; n = 177) or chemical disinfection (Dis; n = 172)], 2 mg estradiol benzoate (Gonadiol; MSD), and 100 μg GnRH (Fertagyl; MSD). On d-3, cows received 25 mg dinoprost (PGF; Lutalyse; Zoetis) and a second PGF treatment on d-2 along with removal of the implant and treatment with 1 mg estradiol cypionate (ECP; Zoetis). All cows received FTAI on d0. A subset of cows (n = 143) was evaluated by ultrasound on d-10, -8, -6, -3, -2, 0, and d5 to identify ovarian structures, and blood samples were collected on d-10, d-3 and d-2 for P4 concentrations by radioimmunoassay. Pregnancy diagnoses were performed at d32 and d60. Statistical analyses were performed using Proc-Mixed for continuing variables and Proc-Glimmix of SAS 9.4 for binomial variables ($P < 0.10$). The treatments did not differ in circulating P4 on d-10 or d-3 but P4 was greater on d-2 in Dis cows. Cows that ovulated to the treatments on d-10 had lower circulating P4 on d-10 (2.0 vs. 3.1 ng/mL) but greater P4 on d-3 (4.0 vs. 2.4 ng/mL), associated with a greater proportion of cows having a CL on d-3 (100 vs. 40%). A greater proportion of ovulating cows on d-10 had a synchronized new follicular wave (97.9 vs. 63.2%) and earlier wave emergence (1.9 vs. 2.6 d), resulting in a lower percentage of cows ovulating a persistent follicle (0.0 vs. 35.7%). More cows without a CL on d-10 showed estrus on d0 compared to cows with a CL on d-10. Treatment (type of P4 insert), CL presence on d-10, and ovulation on d-10 did not affect fertility (pregnancy per AI; P/AI) to the protocol. However, P/AI on Farm A was greater than on Farm B at 32 (40.8 vs. 27.8%) and 60 days (35.8 vs. 24.3%), independent of treatment. In conclusion, the use of P4 inserts produced different circulating P4 patterns during the FTAI protocol but did not affect follicular dynamics, synchronization rate, or P/AI. However, presence of CL at the beginning of the protocol or ovulation at the beginning of the FTAI protocol affected several reproductive variables, such as the time and synchronization of follicular wave emergence, proportion of cows in estrus at the end of the protocol and size of the ovulatory follicle. Beyond that, more overall synchronized cows became pregnant to the FTAI protocol.

Keywords: Hormone; Synchronization; Implant; *Bos taurus*

4.1 Introduction

Fixed-time artificial insemination (FTAI) programs are largely used worldwide and represent an important reproductive management tool to improve reproductive efficiency and profitability of commercial dairy herds (NORMAN et al., 2009). Although many dairies use AI as a way to improve the genetics of their herds with the use of proven sires (VISHWANATH, 2003), there are challenges of maintaining good reproductive performance due to reduced detection of estrus (LOPEZ et al., 2004; WASHBURN et al., 2002) and declining pregnancies per AI (P/AI; BUTLER, 2000; LUCY, 2001; WASHBURN et al., 2002). The use of FTAI programs can reduce labor for managing AI by precisely synchronizing ovulation and have contributed to improvements in reproductive indexes (WILTBANK; PURSLEY, 2014).

Since the first reported FTAI protocol was developed (PURSLEY; MEE; WILTBANK, 1995), there have now been several modifications and improvements (BINELLI et al., 2014, WILTBANK; PURSLEY, 2014), although the main objectives continue to be the same: 1) Synchronization of follicle wave emergence, 2) Synchronization of corpus luteum (CL) function and circulating progesterone (P4), and 3) Synchronization of final ovulation with optimally scheduled FTAI. To achieve these objectives, two major types of pharmaceutical approaches are available: 1) GnRH-based protocols, that use a combination of GnRH analogs at the beginning and at the end of the protocol, followed by one (SOUZA et al., 2008; PURSLEY; MEE; WILTBANK, 1995) or two (BRUSVEEN et al., 2009) prostaglandin $F_2\alpha$ (PGF) treatments, and 2) Estradiol (E2)/P4-based protocols, that use a combination of P4/progestin and E2 esters, usually estradiol benzoate (EB), at the start of the protocol and one (PEREIRA et al., 2013) or two PGF treatments (BINELLI et al., 2014; PEREIRA et al., 2014). These protocols also use E2 esters, EB or estradiol cypionate (ECP), to synchronize ovulation at the end of the protocol.

These two types of hormonal protocols have different advantages and disadvantages. Treatment with GnRH at the beginning of the GnRH-based protocols can induce ovulation of the dominant follicle, if present, leading to initiation of a new follicular wave and formation of a new CL, potentially increasing circulating P4 concentrations during development of the preovulatory follicle wave (PEREIRA et al., 2015). However, many studies have reported that 50% or fewer dairy cows ovulate when GnRH is given at a random stage of the estrous cycle (BILBY et al., 2013; BISINOTTO et al., 2013; GIORDANO et al., 2012b; LOPES et al., 2013). Lack of ovulation to the initial GnRH treatment leads to less than optimal follicle wave synchronization and fertility. On the other hand, the combination of P4 and E2 at the beginning

of the protocol in E2/P4-based protocols leads to a suppression in secretion of gonadotropins (FSH and LH), causing regression of the follicles in the current follicular wave (BÓ et al., 2002; BURKE et al., 1996; CAVALIERI et al., 2003) and initiation of a new follicular wave 3 to 5 days later. Although the protocol can be initiated at any stage of the estrous cycle, almost 30% of the cows do not have emergence of a new follicular wave after the initial E2/P4 treatment leading to ovulation of a larger persistent follicle at the end of the protocol, and this produces lower fertility (MONTEIRO Jr. et al., 2015).

To offset these problems, a combination of GnRH with E2/P4 treatments at the initiation of the FTAI protocol has been evaluated with the encouraging observation of improved fertility in lactating dairy cows submitted to a protocol that lasted 11 days and had two treatments with PGF at the end of the protocol (PEREIRA et al., 2015). However, this initial study did not evaluate the follicular dynamics during the protocol and, in particular, whether ovulation of persistent follicles was avoided with this approach. Moreover, the ovarian physiological responses of cows treated with GnRH plus E2/P4 at the beginning of a protocol has not been tested, especially when using intravaginal inserts with distinct P4 release patterns.

Circulating P4 concentration is determined by a balance between P4 production and P4 metabolism, primarily by the liver (WILTBANK et al., 2014). The importance of P4 for fertility and pregnancy maintenance is well documented and decreased fertility in lactating dairy cows is often associated with decreased concentrations of circulating P4 (WILTBANK et al., 2006). Compared to non-lactating dairy cows and heifers, lactating cows have lower plasma concentrations of P4 (SARTORI et al., 2004) due to high hepatic metabolism of steroid hormones induced by the elevated dry matter/energy intake (SANGSRITAVONG et al., 2002).

Several researchers have reported the importance of adequate concentrations of P4 during preovulatory follicle development, particularly in FTAI programs (BILBY et al., 2013; BISINOTTO et al., 2013; CERRI et al., 2011a, 2011b; CHEBEL et al., 2010; DISKIN et al., 2006; STEVENSON et al., 2006, 2008). Depending on circulating P4 concentrations, the pattern of follicle development can be modified, and low circulating P4 during the growth of the ovulatory follicle is often associated with lower fertility in lactating dairy cows undergoing a FTAI protocol (CERRI et al., 2011a). Low concentration of P4 allows for increased LH pulse frequency which could extend follicular dominance (SAVIO et al., 1993) and compromise oocyte quality due to premature resumption of meiosis (INSKEEP, 2004), and consequently, fertility (BISINOTTO et al., 2010; CUNHA et al., 2008). Adequate circulating P4 during development of the ovulatory follicle is particularly important in the nearly 30% of dairy cows

that are anovular or lack a CL at the beginning of the FTAI protocol (SANTOS et al., 2009; STEVENSON et al., 2008). In these cows, the risk of becoming pregnant is reduced by 30% (BISINOTTO et al., 2010).

For the purposes of our physiological studies in this trial, we chose to compare the autoclaved vs. disinfected 1.9 g P4 implant because of the dramatic differences in the P4 profile throughout the 8 d treatment period, apparent lack of problem with residual P4 depletion, and similarity of residual P4 in the two treatments at the start of reuse of the P4 implant, based on the previous study from our laboratory presented above. Thus, the objectives for this experiment were to compare circulating P4, ovarian dynamics and fertility in lactating dairy cows that were treated with reused 1.9 g intravaginal P4 implants that were previously autoclaved or chemically disinfected as part of a 10-day long E2/P4-based FTAI protocol, combined with GnRH treatment at the beginning of the protocol. The hypotheses for this experiment were that: 1) The combination of GnRH and EB at the beginning of the protocol would produce better synchronization of the wave emergence in cows treated with autoclaved P4 implants, due to the rapid P4 increase after treatment with autoclaved inserts, compared to disinfected P4 inserts; and 2) Elevated P4 concentrations during the FTAI protocol would produce better fertility in cows treated with an autoclaved insert compared to a chemically-disinfected insert.

4.2 Materials and Methods

This experiment was conducted in two commercial dairy farms. The Animal Research Ethics Committee of Escola Superior de Agricultura “Luiz de Queiroz” (ESALQ)/University of São Paulo approved all procedures involving cows in this study.

4.2.1 Cows, housing and diets

For this study, 349 lactating Holstein cows were used (123 primiparous and 226 multiparous). At the beginning of the experiment (d-10), cows averaged (mean ± SD) 163.9 ± 141.86 days in milk (DIM), yielding 35.7 ± 11.31 kg/day of milk, with body condition score (BCS) of 2.9 ± 0.47, lactation number of 2.3 ± 1.37 and number of AI of 2.4 ± 3.08. At Farm A, 161 cows were enrolled (55 primiparous and 106 multiparous) and averaged at the beginning of the experiment (mean ± SD) 127.8 ± 96.63 DIM, yielding 40.5 ± 10.21 kg/day of milk, with

BCS of 2.9 ± 0.50, lactation number of 2.2 ± 1.32 and number of AI of 1.52 ± 1.96. Cows were housed in a cross-ventilated free-stall barn with free access to water and mineral salt and fed *ad libitum* with a TMR diet based on corn silage and Tifton 85 hay as forages, and concentrate based on corn and soybean meal, minerals and vitamins balanced to meet or exceed the nutritional requirements of lactating dairy cows (NRC, 2001). At Farm B, 188 cows were enrolled (68 primiparous and 120 multiparous) and averaged at the beginning of the experiment (mean ± SD) 195.6 ± 166.00 DIM, yielding 31.4 ± 10.54 kg/day of milk, with BCS of 3.0 ± 0.45, lactation number of 2.4 ± 1.41 and number of AI of 3.3 ± 3.62. Cows were housed in a compost bedded pack barn with free access to water and mineral salt and fed *ad libitum* with a TMR diet based on corn silage as forage, and concentrate based on corn and soybean meal, minerals and vitamins balanced to meet or exceed the nutritional requirements of lactating dairy cows (NRC, 2001).

Throughout the experiment, cows in both farms were milked three times daily, 8 hours apart, and all received 500 mg of bovine somatotropin (Lactotropin; Elanco Saúde Animal, São Paulo, Brazil) every 14 days, starting at approximately 60 days postpartum.

4.2.2 Protocols and treatments

Cows were randomly assigned to one of two treatment groups using a completely randomized design of treatments. At the beginning of the FTAI protocol (d-10), the cows received an autoclaved (Aut; n = 177) or disinfected (Dis; n = 172) 8-days used intravaginal P4 implant [CIDR (1.9 g P4), Zoetis], that remained for 8 days. Immediately after P4 insertion, cows were treated with EB [Gonadiol, MSD Saúde Animal, São Paulo, Brazil (2.0 mg, IM)] and GnRH [Fertagyl, MSD Saúde Animal (Gonadorelin, 100 µg, IM)]. At 7 (Day -3) and 8 (d-2) days after implant insertion, dinoprost tromethamine [PGF; Lutalyse, Zoetis (25 mg, IM)] was administered and on d-2, after the withdrawal of the P4 implant, cows received ECP [ECP, Zoetis (1.0 mg, IM)] to synchronize ovulation. Fixed-time AI was performed at d0, 48 hours after ECP and cows were bred with conventional frozen/thawed semen from Holstein sires (Fig. 1).

After treatments on d-2, a subset of 115 cows of Farm A was fitted with a heat detection device (Estrotect, IVP Brasil, São Paulo, Brazil), which remained until d0, at the time of AI.

Cows that had greater than half of the patch coating removed were classified as exhibiting standing estrus.

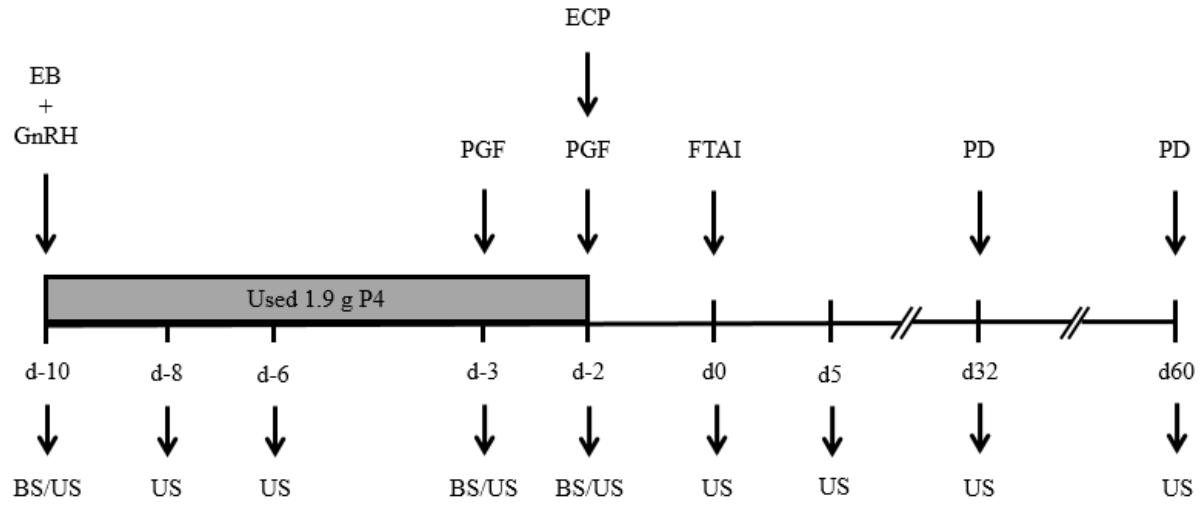

Figure 1 - Diagram of activities for Experiment 2. Cows were submitted to fixed-time artificial insemination (FTAI) protocol, and on d-10 cows were completely randomized receiving a reused autoclaved (Aut) or disinfected (Dis) intravaginal progesterone (P4) device (1.9 g), followed by an intramuscular (IM) injections of 2.0 mg of estradiol benzoate (EB) and 100.0 µg of gonadorelin (GnRH). On d-3 and d-2, 25.0 mg of dinoprost tromethamine (PGF) was administered IM. Also on d-2, the P4 devices were removed, and cows received 1.0 mg of estradiol cypionate (ECP) IM. All cows were inseminated on d0. By ultrasonography, ovulation was confirmed on d5 and pregnancy diagnosis (PD) was performed on d32 and confirmed on d60. US = ovary scanned by ultrasound; and BS = blood sample for serum P4 concentration

4.2.3 BCS, DIM, milk yield and ovarian structures

At experiment enrollment, all cows were scored for body condition using a 1 to 5 point scale (FERGUSON et al., 1994). For this experiment, BCS was categorized as lower BCS (< 2.75) or higher BCS (≥ 2.75) and also categorized by DIM into lower (< 120) or higher (≥ 120 days after calving). Based on parity, milk yield was categorized for primiparous cows (lower ≤ 27.6 kg/day of milk or higher > 27.6 kg/day of milk) and multiparous cows (lower ≤ 31.9 kg/day of milk or higher > 31.9 kg/day of milk) for further analyses.

From a subset of cows (n = 142) of Farm A, ovaries were evaluated using a transrectal ultrasound machine (DP-2200 VET; Mindray, Shenzhen, China) with a 7.5 MHz linear-array transducer on d-10, -8, -6, -3, -2 and 0 of the protocol and on d5 after FTAI. At the beginning of the experiment (d-10), ovaries were evaluated to confirm the presence or absence of a CL and to measure the diameter of the largest follicle. Ovaries were again evaluated on d-3, to confirm the presence or absence of CL and to determine whether CL regression occurred

between d-10 and -3. Ovulation following the treatments on d-10 was recorded on d-8 by the disappearance of any ovulatory follicle and it was confirmed on d-6 by the presence of a new CL. Further evaluations were performed to determine the day of follicle wave emergence and to characterize the future ovulatory follicle growth and size until d0. The size of the follicles and CL was based on the average cross-sectional diameter. On d5, ovulation and number of ovulations were confirmed by detection of CL ipsilateral to the ovary in which ovulatory follicles were present.

4.2.4 Intravaginal P4 devices preparation

The autoclaved and disinfected devices were previously used in lactating dairy cows for 8 days. After removal, the inserts were washed in clean running water, and air dried at room temperature. Prior to use in the experiment, the inserts were autoclaved or disinfected. The protocol used to autoclave the P4 devices was similar to the one described by Cerri et al. (2009). Briefly, the inserts were placed in autoclave bags and autoclaved for 15 minutes at 121ºC and 725 mmHg. For disinfection, the devices were dipped for 15 minutes in 1:2000 diluted quaternary ammonia (CB-30 TA; Ourofino, São Paulo, Brazil) and air dried at room temperature as well.

4.2.5 Blood collection and P4 assay

From a subset of cows (n = 142) from Farm A, blood samples were collected by puncture of the coccygeal vein or artery into 10 mL evacuated tubes (Vacutainer; Becton Dickinson) for P4 measurements, immediately before administration of treatments on d-10, -3 and -2. After collection, samples were placed in ice and transported to the laboratory within 5 hours and kept refrigerated overnight. Blood tubes were centrifuged at 1900 x g for 15 minutes at 4ºC and serum was frozen at -20ºC for further analyses of P4 by a solid-phase radioimmunoassay using a commercial kit (ImmuChemTM Progesterone CT, 07-270105, MP Biomedicals, Santa Ana, CA, USA), according to manufacturer's instruction, except that incubation was done overnight at room temperature. The assay sensitivity was 0.02 ng/mL and the intra-assay coefficient of variation was 6.9%.

4.2.6 Pregnancy diagnosis and reenrollment of previously synchronized cows

Pregnancy diagnosis was determined at 32 days after AI by transrectal ultrasonography (DP-2200 VET; Mindray, Shenzhen, China) of the reproductive tract. Pregnant cows were reconfirmed at 60 days after AI. At each pregnancy diagnoses, pregnancy was only designated if the embryo was identified and had heartbeat. Pregnancy per AI (P/AI) was calculated at d32 and 60 and pregnancy loss between these two evaluations. At any time during the experiment, cows that were diagnosed not pregnant were reenrolled in the experiment for further resynchronization.

4.2.7 Statistical Analysis

Categorical data were analyzed by logistic regression using the GLIMMIX procedure of SAS version 9.4 (SAS/STAT, SAS Institute Inc., Cary, NC) fitting a binary distribution response. The models included the fixed effects of treatment on d-10, parity as primiparous and multiparous, categorized milk yield within parity as below or above the mean value, categorized DIM as below or above 120 DIM, categorized BCS as low or moderate, the interactions between treatments and parity, treatments and categorized milk yield, treatments and categorized DIM and treatments and categorized BCS. The estimates were back-transformed using the ILINK function of SAS to generate the adjusted Tukey percentages, and the results are expressed as least square means ± standard error of means (LSM ± SEM). 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 continuous data such as size of the largest follicle on d-10, size of the largest follicle on d-6, size of the largest follicle on d-3, size of the largest follicle on d-2, size of the largest follicle on d0 and serum P4 concentrations on d-10, d-3 and d-2 were analyzed using the MIXED procedure of SAS version 9.4. Data were tested for normality of residuals using the UNIVARIATE procedure of SAS. The P4 data were analyzed as nonparametric using the Kruskal-Wallis test ordered by the RANK procedure of SAS. The models included the fixed effects of treatment on d-10, parity, categorized milk, categorized DIM, categorized BCS, the interactions between treatments and parity, treatments and categorized milk yield, treatments and categorized DIM and treatments and categorized BCS. The Kenward-Roger method was used to calculate the denominator degrees of freedom to approximate the F-tests in the mixed models. The estimates were back-transformed using the PDIFF function of SAS to generate the adjusted Tukey comparisons of means.

When a treatment outcome was 0 or 100%, we used the Fisher Exact Test using the FREQ procedure of SAS. The results are expressed as LSM ± SEM. For all analyses, only variables with $P < 0.20$ were kept in the final model, unless the variable was essential, such as treatments and their interactions. Differences were considered significant when $P \leq 0.05$, whereas a tendency was defined as $0.10 \geq P > 0.05$.

4.3 Results and Discussion

This Experiment was performed with reused 1.9 g intravaginal P4 implants (previously used for 8 d in lactating dairy cows) that were either autoclaved or chemically disinfected prior to reuse, based on the differences in circulating P4 profiles that were obtained from a previous study from our laboratory. Cerri et al. (2009) compared a new and reused-after-autoclaving 1.38 g P4 implant in a GnRH-based protocol but no previous comparison has been made between P4 implants prepared by the two different disinfection/sterilization techniques that produced such dramatic differences in circulating P4 profiles. In addition, although the combination of GnRH and EB at the beginning of the protocol has been investigated before in an E2/P4-based FTAI protocol (PEREIRA et al., 2015), the follicular dynamics and the effects of used implants in FTAI protocol, prepared by autoclaving vs. chemical-disinfection, had not been previously reported.

In beef cattle, especially in *Bos indicus* cows, disinfected inserts with different days of use have been tested (CREPALDI et al., 2009; SALES et al., 2009) with similar results in P/AI, compared to new inserts. In dairy cattle, Cerri et al. (2009) did not detect differences on fertility when reused, autoclaved implants were compared to new 1.38 g P4 inserts.

In our study, P4 concentrations were not affected by treatments on d-10 (before any treatment) or d-3 (Table 1). Based on P4 profiles of 1.9 g intravaginal implants from a previous study reported in the previous chapter of this thesis, it was expected that cows that received an autoclaved P4 implant would have greater circulating P4 throughout the protocol, particularly during the first 4 days. Unexpectedly, P4 concentrations were slightly but significantly ($P = 0.05$) greater on d-2 in cows with disinfected rather than autoclaved P4 implants. Probably, this difference could be due to a less effective induction of luteolysis in cows receiving disinfected P4 implants. This could be related to the previous observation that CL did not completely regress after one PGF treatment in a relatively high percentage of cows, resulting in reduced

fertility (BRUSVEEN et al., 2009; MONTEIRO Jr. et al., 2015; PEREIRA et al., 2013; SOUZA et al., 2007). Beyond that, this greater circulating P4 on d-2 observed in cows treated with disinfected devices might have lowered LH pulse frequency and, therefore, affected subsequent follicle or CL development or function.

The size of the largest follicle did not differ on d-10, prior to treatment, but there was a significant effect of presence of a CL and an interaction of presence of CL and type of implant on follicle size on d-3 and d-2 (Table 1). Thus, cows with a CL at the start of the protocol, had a smaller size of the subsequent ovulatory follicle than cows without a CL, and follicle size was further reduced by the use of the autoclaved P4 implant rather than the disinfected implant. However, there was no effect of presence of CL at the start of the protocol on ovulatory follicle size (P = 0.70) but the interaction between type of P4 implant and presence of CL was still observed (P = 0.02) with the smallest ovulatory follicle found in cows with a CL at the start of the protocol and treated with the autoclaved P4 (Table 1). It seems likely that high circulating P4 during the protocol in the presence of a CL and an autoclaved P4 implant probably affected ovulatory follicle growth due to reduction in LH pulse frequency (ADAMS et al., 1992).

Alternatively, cows that did not have a CL at the beginning of the protocol, that received an autoclaved P4 implant, had earlier (P = 0.03) wave emergence after treatments on d-10 and subsequently had a larger (P = 0.02) ovulatory follicle diameter on d-2, compared to cows that had a CL and received either type of P4 implant. However, the ovulatory follicle diameter on d0 was not different (Table 1).

Nevertheless, there was no effect of type of P4 implant treatment on percentage of cows that ovulated to the GnRH at the beginning of the protocol (33.1%) or percentage of cows with a CL at the time of PGF treatment (d-3), although, there tended to be a greater proportion of cows with a CL on d-3 when a CL was initially present on d-10, as would be expected (Table 1). If GnRH is given at the beginning of the protocol it may induce ovulation in cows with a follicle greater than 10 mm, causing formation of a new CL with the expected increase in P4 concentrations at the time of PGF treatment (SOUZA et al., 2008). When GnRH was given at the beginning of the E2/P4-based protocol, a greater proportion of cows had CL at the time of PGF treatment, with elevated circulating P4 concentrations and greater fertility (PEREIRA et al., 2015). Although we were not able to detect difference in P/AI between cows having (YES) or not having (NO) CL at d-10 (Table 1) or d-3 (data not shown), the expected elevation in P4 concentrations (either with a CL or greater P4 release from an implant) during preovulatory

follicle development should provide a better endocrine environment for oocyte maturation, potentially leading to improved fertility (BINELLI et al., 2014; CERRI et al., 2011a).

Table 1 - Progesterone (P4) concentrations (mean ± SE), ovarian dynamics and fertility outcomes (LSM ± SE) from lactating dairy cows with or without CL at the beginning of the FTAI protocol, and treated with 8-days used autoclaved (Aut) or disinfected (Dis) 1.9 g intravaginal P4 devices

	CL on d-10				P value		
	No		Yes		Trt	CL	Trt x CL
Itens	**Aut (n = 23)**	**Dis (n = 22)**	**Aut (n = 47)**	**Dis (n = 50)**			
Progesterone concentration, ng/mL							
d-10	0.09 ± 0.46	0.04 ± 0.47	3.60 ± 0.32	4.38 ± 0.32	0.56	< 0.01	0.21
d-3	1.89 ± 0.58	1.76 ± 0.62	3.15 ± 0.39	3.59 ± 0.37	0.95	< 0.01	0.57
d-2	0.53 ± 0.08	0.79 ± 0.08	0.66 ± 0.06	0.73 ± 0.06	0.05	0.50	0.28
Diameter of the follicle, mm							
Largest follicle on d-10	18.2 ± 1.18	16.2 ± 1.23	16.3 ± 0.82	16.0 ± 0.79	0.24	0.30	0.42
Ovulatory follicle on d-3[1]	11.9 ± 0.60^{a}	10.8 ± 0.58^{ab}	9.5 ± 0.37^{b}	10.9 ± 0.45^{a}	0.79	0.03	0.01
Ovulatory follicle on d-2[1]	13.7 ± 0.60^{a}	12.3 ± 0.59^{ab}	11.4 ± 0.34^{b}	12.3 ± 0.41^{b}	0.52	0.02	0.02
Ovulatory follicle on d0[1]	14.9 ± 0.57^{ab}	13.9 ± 0.57^{ab}	13.9 ± 0.37^{b}	15.2 ± 0.44^{a}	0.80	0.70	0.02
Corpus luteum, % (n/n)							
d-3	56.2 ± 10.73 (13/23)	41.0 ± 10.97 (9/22)	63.7 ± 7.28 (30/47)	66.3 ± 6.90 (33/50)	0.51	0.08	0.34
Proportion of cows that ovulated at d-10, %	39.0 ± 10.33 (9/23)	31.7 ± 10.12 (7/22)	31.9 ± 10.12 (15/47)	32.0 ± 6.70 (16/50)	0.69	0.70	0.67
Wave emergence after the beginning of the protocol[1], d	1.6 ± 0.36^{b}	2.3 ± 0.35^{ab}	2.7 ± 0.22^{a}	2.1 ± 0.26^{ab}	0.84	0.13	0.03
Emerged new follicle wave, % (n/n)	73.6 ± 9.44 (17/23)	77.1 ± 9.23 (17/22)	82.9 ± 5.60 (39/47)	66.3 ± 6.86 (33/50)	0.41	0.99	0.21
Showed estrus, %	90.1 ± 6.77 (18/20)	93.8 ± 6.11 (15/16)	72.7 ± 7.29 (29/40)	76.9 ± 6.97 (30/39)	0.60	0.05	0.84
Did not ovulate at d0, %	17.4 ± 7.90 (4/23)	18.2 ± 8.22 (4/22)	12.8 ± 4.87 (6/47)	8.0 ± 3.84 (4/50)	0.73	0.43	0.68
Multiple ovulation, %	26.5 ± 10.82 (5/19)	16.5 ± 9.20 (3/18)	16.5 ± 6.04 (7/41)	24.2 ± 6.75 (11/46)	0.91	0.91	0.30
Ovulated a persistent follicle[2], %	21.2 ± 9.62 (4/19)	27.7 ± 10.9 (5/18)	14.7 ± 5.65 (6/41)	32.2 ± 7.11 (15/46)	0.16	0.81	0.49
Pregnancy/AI, %							
d32	60.9 ± 10.18 (14/23)	45.5 ± 10.62 (10/22)	38.3 ± 7.09 (18/47)	38.0 ± 6.86 (19/50)	0.55	0.34	0.56
d60	56.5 ± 10.34 (13/23)	45.5 ± 10.62 (10/22)	29.8 ± 6.67 (14/47)	34.0 ± 6.70 (17/50)	0.79	0.28	0.55
Pregnancy loss[3], %	7.1 (1/14)	0.0 (0/10)	22.2 (4/18)	10.5 (2/19)	-	0.15	-

[a,b]Values in the same row differ ($P \leq 0.05$)

[A,B]Values in the same row differ ($0.05 < P \leq 0.10$)

[1] Only cows presenting a new follicle wave emergence between d-10 and d5, and a single ovulation were included

[2] Only ovulated cows at the end of the protocol were included

[3] Interaction between treatment and CL on d-10 was not considered

Although no differences were observed in the proportion of cows ovulating at the beginning of the protocol, the earlier wave emergence observed in the Aut group from cows not bearing a CL may be explained by ~18% more cows ovulating in this group on d-10 (Table 1). It is not possible to define in this study if cows ovulated to GnRH or EB, but based on previous data (MELO et al., 2016), several cows in a random phase of the estrous cycle ovulated when EB was used at the beginning of the protocol, in spite of the presence of an intravaginal P4 implant and ~34% ovulated when GnRH was used, also in the presence of a P4 insert. Based on our results, the combination of GnRH and EB at the start of the protocol does not seem to have increased ovulation rate. When cows ovulate in a normal estrous cycle, a new follicular wave starts on the same day of the GnRH-induced gonadotropin surge (SARTORI et al., 2004). However, even with an earlier emergence of a new follicular wave (P = 0.03; Fig. 2A), it is likely that cows ovulating at the beginning of the protocol in this study had delayed wave emergence because of the negative feedback on FSH induced by the high circulating E2 that originated from the EB treatment (SARTORI et al., 2016). On the other hand, most of the non-ovulating cows had later follicle wave emergence, ~3 days after the start of the protocol (P = 0.01; Fig. 2A), which can explain the delay in the day of wave emergence in cows bearing a CL on d-10 from the Aut group, with more cows probably synchronized to EB (Table 1 and Fig. 2A). This finding is in agreement with other authors that showed the expected follicle wave emergence starting after 3 to 5 days after EB treatments (BÓ et al., 1993; MONTEIRO Jr. et al., 2015; SARTORI et al., 2003; SOUZA et al., 2009;). Furthermore, less than 40% of cows ovulated to treatments at the beginning of the protocol, which was unexpectedly low compared to previous studies that reported ~50% of ovulation at the start of GnRH-based protocols that initiated at a random day of the estrous cycle (BILBY et al., 2013; BISINOTTO et al., 2013; GIORDANO et al., 2012b; LOPES et al., 2013), although in agreement with other authors (MELO et al., 2016; MONTEIRO Jr. et al., 2015).

Table 2 - Progesterone (P4) concentrations (mean ± SE), ovarian dynamics and fertility outcomes (LSM ± SE) from lactating dairy cows that ovulated (Yes) or not (No) at the beginning of the FTAI protocol, independent of treatments with 8-days used autoclaved or disinfected 1.9 g intravaginal P4 devices

	No (n = 97)	**Yes (n = 46)**	**P value**
Serum P4 concentration, ng/mL			
d-10	3.14 ± 0.30	2.00 ± 0.43	0.10
d-3	2.40 ± 0.28	3.98 ± 0.39	< 0.01
d-2	0.62 ± 0.04	0.81 ± 0.06	< 0.01
Diameter of the follicle, mm			
Largest follicle on d-10	16.1 ± 0.59	17.2 ± 0.81	0.29
Ovulatory follicle on d-3[1]	10.2 ± 0.33	10.8 ± 0.39	0.22
Ovulatory follicle on d-2[1]	11.9 ± 0.31	12.4 ± 0.36	0.29
Ovulatory follicle on d0[1]	14.3 ± 0.32	14.5 ± 0.37	0.72
Corpus luteum, % (n/n)			
d-10	69.5 ± 4.88 (66/95)	66.3 ± 7.12 (31/47)	0.71
d-3	40.0 (38/95)	100.0 (47/47)	< 0.01
Wave emergence after the beginning of the protocol[2], d	2.6 ± 0.18	1.9 ± 0.22	< 0.02
Emerged new follicle wave, % (n/n)	63.2 ± 5.00 (60/95)	97.9 ± 2.11 (46/47)	< 0.01
Showed estrus, % (n/n)	83.0 ± 4.41 (63/76)	74.4 ± 7.10 (29/39)	0.29
Early ovulation, % (n/n)	8.6 (7/81)	0 (0/37)	0.10
Did not ovulate at d0, %	11.6 ± 3.28 (11/95)	14.9 ± 5.19 (7/47)	0.68
Multiple ovulation on d0, % (n/n)	19.1 ± 4.48 (16/84)	24.8 ± 7.14 (10/40)	0.49
Ovulated a persistent follicle[2], %	35.7 (30/84)	0.0 (0/40)	< 0.01
Pregnancy per AI			
32 d, % (n/n)	41.1 ± 5.05 (39/95)	46.8 ± 7.28 (22/47)	0.63
60 d, % (n/n)	35.8 ± 4.91 (34/95)	42.6 ± 7.21 (20/47)	0.58
Pregnancy loss, % (n/n)	12.8 ± 5.43 (5/39)	9.1 ± 6.19 (2/22)	0.67

[1] Only cows presenting a new follicle wave emergence between d-10 and d5, and a single ovulation were included
[2] Only cows presenting new follicle wave emergence between d-10 and d-5

Independent of treatments or the presence of the CL on d-10, ovulating cows at the beginning of the protocol started the follicle wave emergence 0.7 days earlier ($P < 0.02$), even in the presence of high circulating EB-induced E2 (Table 2). As mentioned above, suppressed circulating FSH is expected when heifers or cows are treated with the combination of P4/progestins and EB (MARTINEZ et al., 2005; O'ROURKE et al., 2000; SARTORI et al., 2003, 2016). However, in one study (RAMOS et al., 2010), crossbred heifers submitted to ovum pick-up (OPU) sessions and treated with P4/progestins, at the time of OPU, only had a marginal effect in suppressing FSH concentrations when treated with EB. Therefore, delayed emergence of a new follicle wave did not occur when EB was given immediately after OPU. Similarly, in this study, if cows ovulated at the beginning of the protocol, EB combined with P4 probably did not efficiently suppress FSH, and therefore, emergence of a new follicular wave was earlier than expected.

Although there was no difference on the proportion of cows with CL at the start of the protocol between cows that ovulated (Yes) or did not ovulate (No) at the beginning of the protocol (~70%; P = 0.71), cows that ovulated at the beginning tended (P = 0.10) to have lower P4 concentrations on d-10 compared to non-ovulating cows (Table 2). Increased ovulation rate in cows with lower circulating P4 was expected, due to the increased magnitude of GnRH-induced LH surge (COLAZO et al., 2008; DIAS et al., 2010; GIORDANO et al., 2012a). Moreover, greater P4 can affect LH receptor expression in granulosa cells (DIAS et al., 2014) and may initiate atresia of a dominant follicle (ADAMS et al., 1992).

Independent of treatments, a greater (P < 0.01) proportion of ovulating cows at the beginning of the protocol had CL and greater (P < 0.01) circulating P4 on d-3 and d-2, compared to non-ovulating cows (Table 2). During the FTAI protocol, two PGF treatments were performed on d-3 and d-2. Because most of the ovulating cows had a new CL or more than one CL on d-3, it is likely that 24 hours after the first treatment with PGF was not enough time to detect a complete regression of the CL and decrease in circulating P4 (BRUSVEEN et al., 2009; PEREIRA et al., 2013; SOUZA et al., 2007).

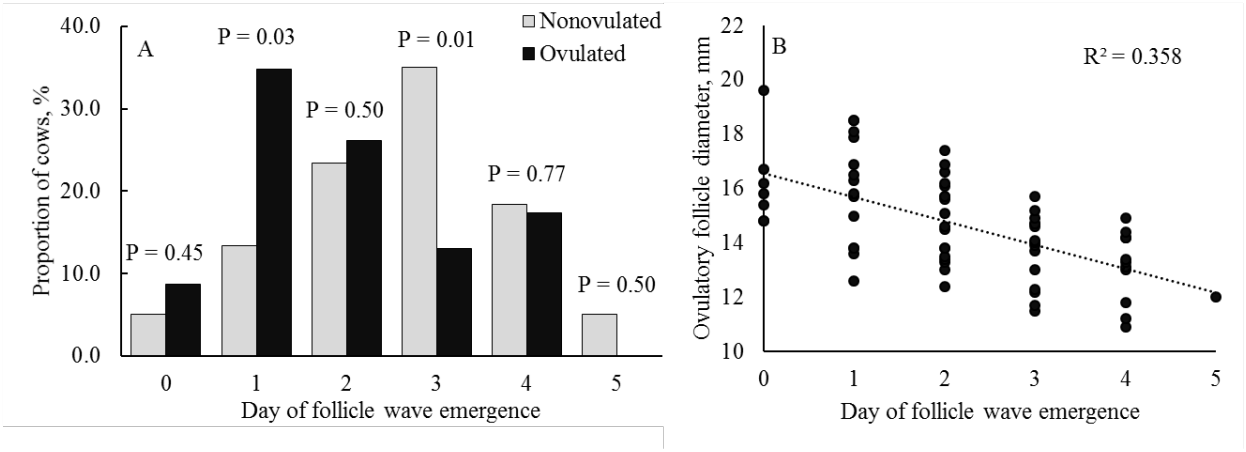

Figure 2 - Proportion of cows that ovulated (46/47) or not (60/95) at the beginning of the protocol in relation to the day of follicle wave emergence (**Fig. A**), and relationship between ovulatory follicle diameter and the day of follicle wave emergence (n = 52; **Fig. B**) in lactating dairy cows

The treatments did not affect (P = 0.41) the percentage of cows emerging a new follicular wave at the beginning of the protocol. On average, 74.7% of the cows emerged a new follicular wave (Table 1 and Table 4). However, when cows ovulated after the start of the protocol, a

greater ($P < 0.01$) proportion of cows emerged a new follicular wave compared to cows not ovulating (Table 2). Similar results were described in a previous study that reported 73.8% of cows had emergence of a new follicular wave in an E2/P4-based protocol (MONTEIRO Jr. et al., 2015). Surprisingly, ~25% of the cows did not have synchronized emergence of a new follicular wave, even when EB was combined with GnRH at the start of the protocol, and ovulated a persistent follicle at the end of the protocol (Table 1 and Table 4). Fertility is compromised when cows ovulate a persistent follicle at the end of the FTAI protocol (MONTEIRO Jr. et al., 2015). Although we were not able to detect differences in P/AI between cows emerging or not emerging a new follicular wave after treatments at the initiation of the FTAI protocol, based on the few number of cows in this study, 33% more cows became pregnant when a new follicle wave emerged. In one of our previous studies, 51% more cows became pregnant when a new follicular wave emerged, compared to cows ovulating a persistent follicle (MONTEIRO Jr. et al., 2015).

On average, 87.3% of the cows ovulated at the end of the protocol after ECP treatment. Beyond deficiencies in synchronizing the follicular wave emergence, E2-esters also failed to induce ovulation at the end of E2/P4-based protocols in some cows (Table 1, Table 2, and Table 4). Likewise, other studies using E2/P4-based FTAI protocols have also shown similar or even greater rates of ovulation failure (MONTEIRO Jr. et al., 2015; PEREIRA et al., 2015). There are distinct pharmacodynamic differences between E2-esters, such as EB and ECP (SOUZA et al., 2005), often used to induce ovulation at the end of E2/P4-based protocols (MONTEIRO Jr. et al., 2015; PEREIRA et al., 2013, 2015). The same dosage of EB, compared to ECP, treatment produced an earlier (16.0 vs. 30.7 hours) and a greater maximum E2 peak (9.6 vs. 3.4 pg/mL), and this could produce a more synchronized ovulation. However, ECP treatment may provide a more prolonged elevation in circulating E2, which may provide a more physiological endocrine environment during proestrus (BINELLI et al., 2014; SOUZA et al., 2005) and one less time for management intervention. Despite different profiles, ovulation rate did not differ between E2-esters and the expected time of the ovulation was also similar (~72 hours after intravaginal P4 implant removal; BARUSELLI et al., 2012). When ECP was compared to GnRH at the end of the E2/P4 FTAI protocol, ovulation rate did not differ between treatments, as well (FERREIRA et al., 2015). Furthermore, P/AI did not differ when EB and ECP were compared at the end of the protocol (MELO et al., 2016). Thus, it seems likely that ECP treatment is an efficient, but imperfect, method for inducing ovulation in this type of protocol. Several factors could compromise ovulation at the end of E2/P4-based protocols, such as the

size of the ovulatory follicle not optimizing response to the E2-GnRH induced LH surge (SARTORI et al., 2001) and increased P4 concentrations near the time of AI (BISINOTTO et al., 2010; BRUSVEEN et al., 2009; GIORDANO et al., 2012a, 2013; SOUZA et al., 2007). We have shown that in Holstein cows, ovulatory capacity was acquired when the follicle size was > 10 mm (SARTORI et al., 2001). Therefore, although the average size of the ovulatory follicle observed in this study was greater than 13 mm in all treatment groups, some cows in each group had smaller follicles at the end of the protocol. Moreover, small elevations in circulating P4 at the time of AI may compromise ovulation and fertility after ECP treatment (MONTEIRO Jr., 2015). Although we did not measure circulating P4 on the day of AI, P4 concentrations were unexpected greater ($P = 0.05$) in cows from the Dis group on d-2, independent of the CL presence on d-10 (Table 1), and greater ($P < 0.01$) in cows ovulating to treatments on d-10 (Table 2). It is likely that the threshold for P4 concentrations near AI is lower when ECP is used as an ovulation inducer compared to GnRH, although the fertility threshold near the time of final GnRH treatment, to induce ovulation, was reported to be between 0.3 and 0.5 ng/mL (BRUSVEEN et al., 2009; GIORDANO et al., 2012b; SOUZA et al., 2007;). Thus, if complete luteolysis has not occurred by the time of AI, ovulation to ECP might be compromised because of the inhibition of the ECP-induced GnRH-LH surge, since the action of E2 at the hypothalamus can be blocked by P4 (RICHTER et al., 2002; ROBINSON et al., 2000).

In this study, overall synchronization was about 66.2%, based on cows that emerged a new follicular wave and ovulated at the end of the protocol (Table 4). In another study, cows were considered synchronized when they did not have a CL on the day of AI, but had a CL 7 days later (> 90%; PEREIRA et al., 2014), which is very close to our findings of 87.3% of cows ovulating to the protocol. Considering only ovulation to the protocol as the gold-standard for synchronization seems to be inadequate, because cows that did not have emergence of a new follicular wave, will ovulate a persistent follicle at the end of the protocol and should not be considered properly synchronized (MELO et al., 2016; MONTEIRO Jr. et al., 2015).

High multiple ovulations were observed in our study. Independent of treatments, presence of CL on d-10, or ovulatory response to treatments on d-10, ~21% of the cows that ovulated at the end of the protocol had multiple ovulation (Table 1 and Table 2). Multiple ovulation is responsible for the high undesired twinning rate in high-producing lactating dairy cows (WILTBANK et al., 2006). Several risk factors are related to multiple ovulation and might account for the high multiple ovulation in this study, such as high milk production (FRICKE;

WILTBANK, 1999; LOPEZ et al., 2005) and low circulating P4 during preovulatory follicle growth (WILTBANK et al., 2012). The higher metabolism of steroid hormones underlies the reduced circulating P4 in lactating cows (SANGSRITAVONG et al., 2002), but interestingly, even cows that ovulated on d-10, and therefore had greater ($P < 0.01$) circulating P4 concentrations on d-3, compared to non-ovulating cows, also had elevated multiple ovulation (24.8%; Table 2). Although many risk factors may be involved in the occurrence of high multiple ovulation and twinning rate (DEL RIO et al., 2007), it is important to note that CL regression between d-10 and d-3 in our study was greater than 50% (data not shown), probably due to the use of EB at the initiation of the protocol (MELO et al., 2016; MONTEIRO Jr., 2015). This phenomenon could underlie, at least in part, the high incidence of multiple ovulation observed in the present study.

Pregnancy per AI on d32 and d60, or pregnancy loss were not affected by treatments, CL presence on d-10, or ovulatory response to treatments at the beginning of the protocol (Table 1 and Table 2). Although we were not able to detect differences in fertility, independent of treatments, a greater ($P = 0.05$) proportion of cows without a CL on d-10 showed estrus at the end of the protocol, which could be related to the numerically greater P/AI on d32, d60 and lower pregnancy loss (Table 1). Expression of estrus during an E2/P4-based FTAI protocol has been found to increase fertility and reduce pregnancy loss (CERRI et al., 2004; GALVÃO et al., 2004; PANCARCI et al., 2002; PEREIRA et al., 2014; SOUZA et al., 2007). Displaying estrus at the end of an E2/P4-based protocol may be related to reduced P4 concentrations near AI and to increased E2 during proestrus due to E2-esters plus the endogenous E2 from the ovulatory follicle (PEREIRA et al., 2014). This prolonged exposure to E2 during proestrus may underlie the increased fertility and reduced pregnancy loss in cows displaying estrus, which can alter uterine gene and protein expression and provide a better environment for pregnancy maintenance (BINELLI et al., 2014). Moreover, overall synchronized cows had greater ($P < 0.01$) fertility, compared to cows not synchronized to the protocol (Table 4).

Finally, fertility between farms was compared and treatments did not affect P/AI or pregnancy loss (Table 3). However, P/AI was greater ($P = 0.02$) in Farm A compared to Farm B and this may have been related to differences in management factors, such as cow-handling, overcrowding, and cow comfort (SCHEFERS et al., 2010), nutritional factors, such as BCS (CARVALHO et al., 2014), or disease challenges (RIBEIRO et al., 2013).

Table 3 - Fertility outcomes (LSM ± SE) from lactating dairy cows treated with 8-days used autoclaved (Aut) or disinfected (Dis) 1.9 g intravaginal P4 devices during a FTAI protocol on two farms

Variable	**Farm A**		**Farm B**		**P value**		
	Aut	**Dis**	**Aut**	**Dis**	**TRT**	**Farm**	**TRT x Farm**
	Adjusted proportion ± SE (n/n)						
Pregnancy/AI, %							
d32	42.0 ± 5.82 (34/80)	39.5 ± 5.74 (32/81)	24.2 ± 4.58 (23/97)	31.5 ± 5.24 (27/91)	0.58	0.02	0.33
d60	34.6 ± 5.52 (28/80)	37.1 ± 5.60 (30/81)	21.0 ± 4.29 (20/97)	27.6 ± 4.96 (24/91)	0.35	0.03	0.61
Pregnancy loss, %	16.7 ± 6.49 (6/34)	5.6 ± 3.97 (2/32)	12.6 ± 6.96 (3/23)	11.5 ± 6.30 (3/27)	0.29	0.72	0.37

Table 4 - Fertility outcomes (LSM ± SE) from lactating dairy cows responding (Yes) or not (No) to the FTAI protocol

	Percentage of cows with response[1], %	P/AI, %		P value
		Yes	No	
Emerged new wave[2]	74.7 (106/142)	41.5 (44/106)	27.8 (10/36)	0.15
Ovulated to protocol[3]	87.3 (124/142)	43.6 (54/124)	0.0 (0/18)	< 0.01
Overall synchronization[4]	66.2 (94/142)	46.8 (44/94)	20.8 (10/48)	< 0.01

[1]Percentage of cows presenting a new follicle wave emergence, or were detected in estrus, or ovulated at the end of the protocol, or had an overall synchronization of the FTAI protocol
[2] Cows presenting a new follicle wave emergence between d-10 and d-5
[3] Cows ovulating at the end of the protocol between d-0.5 and d1.5
[4] Cows were considered synchronized to the FTAI protocol when they had new follicle wave emergence between d-10 and d-5, and ovulated between d-0.5 and d1.5

4.4 Conclusions

The use of P4 inserts presenting different releasing patterns during the FTAI protocol did not affect follicular dynamics, synchronization rate or P/AI. However, presence of CL at the beginning of the protocol or ovulation at the beginning of the FTAI protocol affected several reproductive variables, such as the time and synchronization of the follicular wave emergence, proportion of cows in estrus at the end of the protocol and size of the ovulatory follicle. Beyond that, more overall synchronized cows became pregnant to the FTAI protocol.

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5 GENERAL CONCLUSIONS

Fixed-time AI programs are largely used worldwide, in beef and in dairy cattle. Since the first GnRH-based protocol (Ovsynch) was developed, many modifications were performed during the last years, generating a variety of protocols to be used in different situations with a combination of several hormones at the beginning and at the end of the protocol. This study aimed to verify the efficacy of different hormonal combinations during the E2/P4-based protocol.

In the first study, which investigated the effects of the combinations of GnRH or EB at the beginning and EB or ECP at the end of the protocol, the interactions among treatments did not affect P/AI or physiology. However, GnRH at the beginning of the protocol increased P4 concentrations and avoided CL regression during the protocol, which occurs in a great proportion of cows when EB is used at the beginning, and improved fertility. Pharmacodynamic differences between EB and ECP should benefit both hormonal treatments at the end of the protocol and may explain why we were not able to detect any effect on fertility.

Based on the results of the second study, autoclaving process before reuse of 8-days used intravaginal devices containing different loads of P4 increased circulating P4 concentrations in non-lactating Holstein cows during 8-days of treatment compared to disinfected, and similar to new. Although circulating P4 concentration from autoclaved 8-days used intravaginal 1.0 g P4 device was similar to new, both differed from disinfected 8-days used intravaginal device. For 1.9 g intravaginal P4 device, circulating P4 concentration was greater for new compared to disinfected 8-days used device. However, circulating P4 concentration from autoclaved 8-days used intravaginal device was greater than both inserts.

Finally, in the third study it was shown that 8-days used intravaginal 1.9 g P4 device can be used in lactating dairy cows without any negative effects on fertility, either autoclaved or disinfected before reuse. However, the expected greater circulating P4 concentrations from autoclaved 8-days used intravaginal 1.9 g device, compared to disinfected, did not improve fertility or affect physiology during the E2/P4-based FTAI protocol that combined EB and GnRH at the beginning. Although not compared, several cows still regress the CL during the protocol, anticipate emergence of a new follicle wave, fail to emerge a new wave, or fail to ovulate at the end of the protocol even with the combination of EB and GnRH at the beginning of the protocol. Presence of CL and ovulation to treatments at the beginning of the protocol remain the main factors affecting fertility or the physiological responses to a FTAI protocol.