Follicular dynamics, circulating progesterone, and fertility in Holstein cows synchronized with reused intravaginal progesterone implants that were sanitized by autoclave or chemical disinfection

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ABSTRACT

This experiment aimed to compare circulating progesterone (P4), follicular dynamics, and fertility during reuse of intravaginal P4 implants that were sanitized by autoclave or chemical disinfection in lactating Holstein cows submitted to fixed-time artificial insemination (FTAI). For this, 123 primiparous and 226 multiparous cows from 2 farms, averaging (mean $\pm$ standard deviation) 163.9 $\pm$ 141.9 d in milk, 35.7 $\pm$ 11.3 kg of milk/d, and a body condition score of 2.9 $\pm$ 0.5, were enrolled in the study. Cows were randomly assigned to 1 of 2 treatments using a completely randomized design and each cow received a reused implant (1.9 g of P4; previously used for 8 d) that was either autoclaved (AUT; n = 177) or chemically disinfected (CHEM; n = 172) on d –10. Also on d –10, cows received 2 mg of estradiol benzoate and 100 μ g of GnRH. On d –3, cows received 25 mg of dinoprost (PGF_{2 α}). A second PGF_{2 α} was given on d –2, along with 1 mg of estradiol cypionate and P4 implant removal. Cows received FTAI on d 0. A subset of cows (n = 143) was evaluated by ultrasound on d –10, –8, –6, –3, –2, 0, and 5 to identify ovarian structures, and blood was sampled on d –10, –3, and –2 for P4 concentrations by RIA. Pregnancy diagnoses were performed at d 32 and 60. Statistical analyses were performed using PROC-MIXED for continuous variables and PROC-GLIMMIX of SAS 9.4 (SAS Institute Inc., Cary, NC) for binomial variables. The treatments did not differ in circulating P4 on d –10 or –3, but P4 was greater on d –2 in CHEM cows. Ovulation to the treatments on d –10 was associated with lower circulating P4 on d –10 (2.0 vs. 3.1 ng/mL) and resulted in greater P4 on d –3 (4.0 vs. 2.4 ng/mL) and more cows with a corpus luteum on d –3 (100 vs. 40%)

than nonovulating cows. Cows that ovulated to d –10 treatments were more likely to have a synchronized new follicular wave (97.9 vs. 63.2%) and had an earlier wave emergence (1.9 vs. 2.6 d), resulting in less cows ovulating a persistent follicle (0.0 vs. 35.7%). Type of P4 implant, corpus luteum presence on d –10, and ovulation to d –10 treatments did not affect fertility (pregnancy per AI; P/AI). However, P/AI on farm A was greater than on farm B at 32 (40.8 vs. 27.8%) and 60 d (35.8 vs. 24.3%), independent of treatment. In conclusion, P4 implants with different P4 release patterns did not produce detectable differences in follicular dynamics, synchronization rate, or P/AI. Nevertheless, presence of corpus luteum or ovulation at the beginning of the FTAI protocol affected reproductive variables, such as timing and synchronization of follicular wave emergence, and size of the ovulatory follicle. Beyond that, more overall synchronized cows became pregnant to the FTAI protocol.

Key words: hormone, synchronization, device, *Bos taurus*

INTRODUCTION

Fixed-time artificial insemination (FTAI) programs are widely 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), challenges exist for maintaining good reproductive performance due to reduced detection of estrus (Washburn et al., 2002; Lopez et al., 2004) 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 and Pursley, 2014).

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Since the first reported FTAI protocol was developed (Pursley et al., 1995), several modifications and improvements have been documented (Binelli et al., 2014; Wiltbank and Pursley, 2014); however, 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, 2 major types of pharmaceutical approaches are available: (1) GnRH-based protocols, which use a combination of GnRH analogs at the beginning and at the end of the protocol, followed by 1 (Pursley et al., 1995; Souza et al., 2008) or 2 (Brusveen et al., 2009; Wiltbank et al., 2015) PGF_{2α} treatments; and (2) estradiol (E2)/P4-based protocols, which use a combination of P4/progestin and E2 esters, usually estradiol benzoate (EB), at the start of the protocol and 1 (Pereira et al., 2013a,b) or 2 PGF_{2α} treatments (Binelli et al., 2014; Pereira et al., 2015). These protocols also use E2 esters, EB or estradiol cypionate (EC), to synchronize ovulation at the end of the protocol.

These 2 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 (Giordano et al., 2012b; Bilby et al., 2013; Bisinotto et al., 2013; Lopes et al., 2013; Melo et al., 2016). 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 (Burke et al., 1996; Bó et al., 2002; Cavalieri et al., 2003) and initiation of a new follicular wave 3 to 5 d 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, which produces lower fertility (Monteiro 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 d and had 2

treatments with PGF_{2α} 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.

Several researchers have reported the importance of adequate concentrations of P4 during preovulatory follicle development, particularly in FTAI programs (Diskin et al., 2006; Stevenson et al., 2006, 2008; Chebel et al., 2010; Cerri et al., 2011a,b; Bilby et al., 2013; Bisinotto et al., 2013). 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., 2011b). Low concentration of P4 allows for increased LH pulse frequency, which could extend follicular dominance (Savio et al., 1993), compromise oocyte quality, possibly due to premature resumption of meiosis (Revah and Butler, 1996; Inskeep, 2004), and, consequently, reduce fertility (Cunha et al., 2008; Bisinotto et al., 2010). 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 (Stevenson et al., 2008; Santos et al., 2009; Melo et al., 2016). In these cows, the risk of becoming pregnant is reduced by 30% (Bisinotto et al., 2010).

For the purposes of our physiological studies, we chose to compare the autoclaved versus chemically disinfected, reused 1.9-g P4 implants because of the dramatic differences in the P4 profile throughout the 8-d treatment period, as shown in our previous study (Melo et al., 2018). In this previous study, the average circulating P4 was greater ($P < 0.05$) for the autoclaved, reused controlled internal drug release (CIDR; 1.67 ± 0.06 ng/mL) than the new CIDR (1.49 ± 0.07), and both were greater than the chemically disinfected CIDR (1.21 ± 0.05). Thus, we hypothesized that the greater circulating P4 that results from using autoclaved compared with chemically disinfected P4 implants would alter the patterns of follicle growth and these changes would be associated with an improvement in fertility. Thus, our objectives were to compare circulating P4, ovarian dynamics, and fertility in lactating dairy cows treated with reused 1.9-g intravaginal P4 implants that were previously autoclaved or chemically disinfected as part of an FTAI protocol that combined GnRH and EB treatment at the beginning of the protocol.

MATERIALS AND METHODS

This experiment was conducted in 2 commercial dairy farms. The Animal Research Ethics Committee of Escola Superior de Agricultura “Luiz de Queiroz”/University of São Paulo approved all procedures involving cows in this study.

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 $\pm$ SD) 163.9 $\pm$ 141.86 DIM, yielding 35.7 $\pm$ 11.31 kg of milk/d, with BCS of 2.9 $\pm$ 0.47 (Ferguson et al., 1994), lactation number of 2.3 $\pm$ 1.37, and AI number of 2.4 $\pm$ 3.08 (approximately 35% of AI were first postpartum AI, and ~30% of the cows enrolled were treated only once). At farm A, 161 cows were enrolled (55 primiparous and 106 multiparous) and averaged at the beginning of the experiment (mean $\pm$ SD) 127.8 $\pm$ 96.63 DIM, yielding 40.5 $\pm$ 10.21 kg of milk/d, with BCS of 2.9 $\pm$ 0.50, lactation number of 2.2 $\pm$ 1.32, and AI number of 1.52 $\pm$ 1.96. Cows were housed in a cross-ventilated freestall 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, concentrate based on corn and soybean meal, and 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 $\pm$ SD) 195.6 $\pm$ 166.00 DIM, yielding 31.4 $\pm$ 10.54 kg of milk/d, with BCS of 3.0 $\pm$ 0.45, lactation number of 2.4 $\pm$ 1.41, and AI number of 3.3 $\pm$ 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, concentrate based on corn and soybean meal, and 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 3 times daily, 8 h apart, and all received 500 mg of recombinant bST (Lactotropin; Elanco Saúde Animal, São Paulo, Brazil) every 14 d, starting at approximately 60 d postpartum.

Protocols and Treatments

Cows were randomly assigned to 1 of 2 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

chemically disinfected (CHEM; n = 172) 8-d used intravaginal P4 implant (CIDR, Zoetis, São Paulo, Brazil; 1.9 g of P4) that remained for 8 d. Immediately after P4 insertion, cows were treated with EB (Gonadiol, MSD Saúde Animal, São Paulo, Brazil; 2.0 mg of EB i.m.) and GnRH (Fertagyl, MSD Saúde Animal, 100 μ g of gonadorelin i.m.). At 7 (d -3) and 8 (d -2) d after implant insertion, PGF_{2 α} (Lutalyse, Zoetis, 25 mg of dinoprost tromethamine i.m.) was administered, and on d -2, after withdrawal of the P4 implant, cows received EC (ECP, Zoetis; 1.0 mg of EC i.m.) to synchronize ovulation. Fixed-time AI was performed at d 0, 48 h after EC administration, and cows were bred with conventional frozen/thawed semen from Holstein sires (Figure 1).

After treatments on d -2, a subset of 115 cows from farm A received a heat detection device (Estrotest, IVP Brasil, São Paulo, Brazil), which remained until d 0, the time of AI. Cows that had greater than half of the patch coating removed were classified as exhibiting standing estrus.

BCS, DIM, Milk Yield, and Ovarian Structures

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

From the majority of the cows (n = 142) on 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 d 5 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 d 0. The size of the follicles and CL was based on the average cross-sectional diameter. On d 5, ovulation and number of ovulations were confirmed by detection of a

Figure 1. Diagram of activities. Cows were submitted to a fixed-time artificial insemination (FTAI) protocol, in which on d -10 they were randomized to receive either a reused autoclaved (AUT) or chemically disinfected (CHEM) intravaginal progesterone (P4) implant (1.9 g), followed by an intramuscular treatment with 2.0 mg of estradiol benzoate (EB) and 100.0 µg of gonadorelin (GnRH). On d -3 and -2, 25.0 mg of dinoprost tromethamine (PGF) was administered i.m. Also on d -2, the P4 implants were removed and cows received 1.0 mg of estradiol cypionate (ECP) i.m. All cows were inseminated on d 0. By ultrasonography, ovulation was confirmed on d 5 and pregnancy diagnosis (PD) was performed on d 32 and confirmed on d 60. US = ovary evaluated using ultrasound; BS = blood sample for serum P4 concentration.

CL ipsilateral to the ovary in which ovulatory follicles were present.

Intravaginal P4 Implants Preparation

The autoclaved and chemically disinfected implants were previously used in lactating dairy cows for 8 d. After removal, the implants were washed in clean running water and air-dried at room temperature. Prior to use in the experiment, the implants were autoclaved or chemically 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 min at 121°C and 725 mmHg. For chemical disinfection, the implants were dipped for 15 min in 1:2,000 diluted quaternary ammonia (CB-30 TA; Ourofino, São Paulo, Brazil) and air-dried at room temperature.

Blood Collection and P4 Assay

From most of the cows (n = 142) on farm A, blood samples for P4 measurements were collected by puncture of the coccygeal vein or artery into 10-mL evacuated tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) 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 h and kept refrigerated overnight. Blood tubes were

centrifuged at $1,900 \times g$ 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 (ImmuChem Progesterone CT, 07-270105, MP Biomedicals, Santa Ana, CA), according to the manufacturer's instruction, except for incubation. Briefly, after bringing standards from the kit, samples, coated tubes, and progesterone I125 to room temperature, 100 µL of samples and standards were pipetted to the tubes. When all samples have been pipetted, 1.0 mL of progesterone I125 was added to all tubes and vortexed briefly. Tubes were incubated overnight at room temperature (instead of in a water bath at 37°C for 2 h) and then aspirated and counted in a gamma counter. The assay sensitivity was 0.02 ng/mL and the intra-assay coefficient of variation was 6.9%.

Pregnancy Diagnosis and Reenrollment of Previously Synchronized Cows

Pregnancy diagnosis was determined at 32 d after AI by transrectal ultrasonography (DP-2200 VET; Mindray) of the reproductive tract. Pregnant cows were reconfirmed at 60 d after AI. At each pregnancy diagnoses, pregnancy was only designated if the embryo was identified and had heartbeat. Pregnancy per AI was calculated at d 32 and 60, and pregnancy loss was recorded between these 2 evaluations. Cows that were diagnosed not pregnant at any time during the experi-

ment were reenrolled in the experiment for further re-synchronization.

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 (primiparous and multiparous), categorized milk yield within parity (below or above the mean value), categorized DIM (below or above 120 DIM), categorized BCS (low or moderate), as well as 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 squares means $\pm$ standard error of means. 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 d 0, 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 yield, categorized DIM, categorized BCS, farm, and the interactions between treatments and parity, treatments and categorized milk yield, treatments and categorized DIM, treatments and categorized BCS, and treatments and farm. 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 PDIF 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 least squares means $\pm$ standard error of the means. 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 $0.10 \geq P > 0.05$.

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 before reuse, based on the differences in circulating P4 profiles that were obtained from a previous study from our laboratory (Melo et al., 2018). 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 2 different disinfection/sanitization 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 versus chemical disinfection, had not been previously reported.

In beef cattle, especially in *Bos indicus* cows, chemically disinfected implants with different days of use have been compared with new implants (Crepaldi et al., 2009; Sales et al., 2009), with similar results in P/AI. In dairy cattle, Cerri et al. (2009) did not detect differences in fertility when reused, autoclaved implants were compared with new 1.38-g P4 implants. In our study, P4 concentrations were not affected by treatments on d -10 (before any treatment) or -3 (Table 1). Based on P4 profiles of 1.9-g intravaginal implants from a previous study (Melo et al., 2018) it was expected that cows that received an autoclaved P4 implant would have greater circulating P4 throughout the protocol, particularly during the first 4 d. Unexpectedly, P4 concentrations were slightly but significantly ($P = 0.05$) greater on d -2 in cows with chemically disinfected rather than autoclaved P4 implants. Possibly, this unexpected difference could be due to a less effective induction of luteolysis in cows receiving chemically disinfected P4 implants. This could be related to the previous observation that CL did not completely regress after 1 PGF_{2α} treatment in a relatively high percentage of cows, resulting in reduced fertility (Souza et al., 2007; Brusveen et al., 2009; Pereira et al., 2013b; Monteiro et al., 2015). Beyond that, this greater circulating P4 on d -2 observed in cows treated with chemically disinfected implants 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, before treatment, but we noted 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

Table 1. Progesterone (P4) concentrations (mean $\pm$ SEM), ovarian dynamics, and fertility outcomes (LSM $\pm$ SEM) from lactating dairy cows with or without corpus luteum (CL) at the beginning of the fixed time AI protocol, and treated with previously used 1.9-g intravaginal P4 implants that were sanitized by autoclave (AUT) or chemical disinfection (CHEM)

Item	CL on d -10				P-value	
	No		Yes		Treatment	Treatment × CL
	AUT (n = 23)	CHEM (n = 22)	AUT (n = 47)	CHEM (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	0.21
d -3	1.89 ± 0.58	1.76 ± 0.62	3.15 ± 0.39	3.59 ± 0.37	0.95	0.57
d -2	0.53 ± 0.08	0.79 ± 0.08	0.66 ± 0.06	0.73 ± 0.06	0.05	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	0.42
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	0.01
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
Ovulatory follicle on d 0 ¹	14.9 ± 0.57 ^{ab}	13.9 ± 0.57 ^{ab}	13.9 ± 0.37 ^b	15.2 ± 0.44 ^a	0.80	0.02
Corpus luteum, % (no./no.)						
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	0.34
Proportion of cows that ovulated to treatments on d -10, % (no./no.)	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	0.67
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	0.03
Emerged new follicle wave, % (no./no.)	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	0.21
Showed estrus, % (no./no.)	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	0.84
Did not ovulate at d 0, % (no./no.)	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	0.68
Multiple ovulation, % (no./no.)	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	0.30
Ovulated a persistent follicle, ² % (no./no.)	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	0.49
Pregnancy/AI, % (no./no.)						
d 32	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	0.56
d 60	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
Pregnancy loss, ³ % (no./no.)	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 with different superscripts differ ($P \leq 0.05$).

¹Only cows with an emergence of a new follicle wave between d -10 and 5 and a single ovulation were included.

²Only cows that ovulated at the end of the protocol were included.

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

−2 (Table 1). Thus, cows with a CL at the start of the protocol had a smaller 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 chemically disinfected implant. However, we found 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 sanitization 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 the presence of a CL combined with the greater release of P4 from an autoclaved P4 implant produced greater circulating P4 concentrations that could affect ovulatory follicle growth due to a reduction in LH pulse frequency (Adams et al., 1992; Bergfeld et al., 1995, 1996).

Alternatively, cows that did not have a CL at the beginning of the protocol and 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 with cows that had a CL and received either type of P4 implant. However, the ovulatory follicle diameter on d 0 was not different (Table 1).

Nevertheless, we observed no effect of type of P4 implant treatment on the 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_{2α} 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_{2α} 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_{2α} 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 −3 (data not shown), the expected elevation in P4 concentrations (either with a CL or greater P4 release from an implant) during pre-ovulatory follicle development should provide a better endocrine environment for oocyte maturation, potentially leading to improved fertility (Cerri et al., 2011b; Binelli et al., 2014). Nevertheless, the lack of treatment difference in P/AI for cows with or without a CL may indicate that the increased P4 from the autoclaved implant may be insufficient to produce physiologically significant changes in circulating P4 in cows either

with or without a CL. Use of a single new CIDR or autoclaved, reused CIDR did not produce luteal phase concentrations of circulating P4 in high-producing lactating dairy cows without a CL, as they were initially developed for nonparous dairy heifers (300–400 kg of BW) in New Zealand (Macmillan et al., 1991; Melo et al., 2018). High-producing dairy cows without a CL require 2 of these implants to achieve sufficient circulating P4 to optimize fertility (Padula and Macmillan, 2006; Bisinotto et al., 2013; Pereira et al., 2017).

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 whether cows ovulated to GnRH or EB; however, 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 intra-vaginal 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 ($P = 0.03$; Figure 2A), it is likely that cows ovulating at the beginning of the protocol in our 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 nonovulating cows had later follicle wave emergence, ~3 d after the start of the protocol ($P = 0.01$; Figure 2A), which might 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 Figure 2A). This finding is in agreement with other authors that showed the expected follicle wave emergence starting at 3 to 5 d after EB treatments (Bó et al., 1995; Sartori et al., 2003; Souza et al., 2009; Monteiro et al., 2015). Furthermore, less than 40% of cows ovulated to treatments at the beginning of the protocol, which was unexpectedly low compared with some previous studies that reported ~50% ovulation at the start of GnRH-based protocols that were initiated on a random day of the estrous cycle (Giordano et al., 2012b; Bilby et al., 2013; Bisinotto et al., 2013; Lopes et al., 2013), although the low ovulatory response is in agreement with other authors (Monteiro et al., 2015; Melo et al., 2016). It seems possible that this dose of GnRH (100 µg of gonadorelin) may not elicit a suffi-

Figure 2. (A) Proportion of cows that ovulated (46/47) or not (60/95) at the beginning of the protocol and emerged a new follicular wave in relation to the day of follicle wave emergence. (B) Relationship between ovulatory follicle diameter and the day of follicle wave emergence in lactating dairy cows ($n = 52$).

cient LH surge. Previous research has shown differences between GnRH products in ovulation efficacy (Martínez et al., 2003; Souza et al., 2009) and that a higher dose of GnRH (200 µg) can dramatically increase the magnitude of the LH surge, which may be particularly important in cows with greater P4 concentrations (Souza et al., 2009; Giordano et al., 2012a).

Independent of treatments or the presence of the CL on d -10, ovulating cows at the beginning of the protocol had follicle wave emergence 0.7 d earlier than non-ovulating cows ($P < 0.02$), even in the presence of high circulating EB-induced E2 (Table 2). As mentioned, suppressed circulating FSH is expected when heifers or cows are treated with the combination of P4/progestins and EB (O'Rourke et al., 2000; Sartori et al., 2003; Martínez et al., 2005). However, Ramos et al. (2010) submitted crossbred heifers to ovum pick-up (OPU) sessions and treated them with P4/progestins at the time of OPU, but only reported a marginal suppression in FSH concentrations in response to treatment with EB; therefore, delayed emergence of a new follicle wave did not occur when EB was given immediately after OPU. Similarly, in our 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 we found no difference in 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 with nonovulating cows (Table 2). Increased ovulation rate

in cows with lower circulating P4 was expected due to the increased magnitude of the 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 -2 compared with nonovulating cows (Table 2). During the FTAI protocol, 2 PGF_{2α} treatments were performed on d -3 and -2. Because most of the ovulating cows had a new CL or >1 CL on d -3, it is likely that 24 h after the first treatment with PGF_{2α} was insufficient to detect complete regression of the CL and basal circulating P4 (Souza et al., 2007; Brusveen et al., 2009; Pereira et al., 2013b).

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 3); however, when cows ovulated after the start of the protocol, a greater ($P < 0.01$) proportion of cows emerged a new follicular wave compared with cows not ovulating (Table 2). Similar results were described in a previous study that reported that 73.8% of cows had emergence of a new follicular wave in an E2/P4-based protocol (Monteiro et al., 2015). Surprisingly, ~25% of the cows in our study did not have synchronized emergence of a new follicular wave, even when EB was combined with GnRH at the start of the protocol, and these cows subsequently ovulated a persistent follicle at the end of the protocol (Table 1 and Table 3). Fertility

Table 2. Progesterone (P4) concentrations (mean $\pm$ SEM), ovarian dynamics, and fertility outcomes (LSM $\pm$ SEM) from lactating dairy cows that ovulated (Yes) or did not ovulate (No) at the beginning of the fixed time AI protocol, independent of treatments with an autoclaved or chemically disinfected reused 1.9-g intravaginal P4 implant

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

¹Only cows with emergence of a new follicle wave between d -10 and -5 and a single ovulation were included.

²Data were analyzed by the Fisher Exact Test.

³Only cows with emergence of a new follicle wave between d -10 and -5.

is compromised when cows ovulate a persistent follicle at the end of the FTAI protocol, with 51% more pregnancies in cows that had emergence of a new follicular wave compared with cows ovulating a persistent follicle (Monteiro et al., 2015). In our study, 33% more cows became pregnant when a new follicle wave emerged, although this did not reach statistical significance.

In addition to the problem with lack of emergence of a new follicular wave, 12.7% of cows failed to ovulate to the EC treatment at the end of the protocol. Likewise, other studies using E2/P4-based FTAI protocols have also shown similar or even greater rates of ovulation

failure (Pereira et al., 2013b; Monteiro et al., 2015). Distinct pharmacodynamic differences exist between E2-esters, such as EB and EC (Souza et al., 2005), often used to induce ovulation at the end of E2/P4-based protocols (Pereira et al., 2013a, 2015; Melo et al., 2016). After treatment of cows with the same dosage of EB, compared with EC, EB treatment produced an earlier increase (16.0 vs. 30.7 h) and a greater maximum E2 peak (9.6 vs. 3.4 pg/mL). This pattern of E2 increase could produce a more synchronized ovulation. On the other hand, EC treatment may provide a more prolonged elevation in circulating E2, which may provide a more

Table 3. Fertility outcomes from lactating dairy cows that were synchronized (Yes) or not synchronized (No), based on emergence of a new follicular wave or ovulation at the end of the fixed time AI protocol

Item	Cows with response, ¹ % (no./no.)	Pregnancy per AI, % (no./no.)		P-value
		Yes	No	
Emerged new wave ²	74.6 (106/142)	41.5 (44/106)	27.8 (10/36)	0.15
Ovulated to protocol ³	87.3 (124/142)	43.5 (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

¹Percentage of cows with the given response (emergence of a new follicle wave, ovulation at the end of the protocol, or overall synchronization to the fixed time AI protocol).

²Cows with emergence of a new follicle wave between d -10 and -5.

³Cows ovulating at the end of the protocol between d -0.5 and 1.5.

⁴Cows were considered synchronized to the fixed time AI protocol when they had new follicle wave emergence between d -10 and -5, and ovulation between d -0.5 and 1.5.

physiological endocrine environment during proestrus (Souza et al., 2005; Binelli et al., 2014) and requires fewer management interventions. Other studies have shown that, despite these differences in circulating E2 profiles, ovulation rate did not differ between E2 esters and the expected time of the ovulation was also similar (~72 h after intravaginal P4 implant removal; Baruselli et al., 2012). When EC was compared with GnRH at the end of the E2/P4 FTAI protocol, ovulation rate also did not differ between treatments (Ferreira et al., 2017). Furthermore, P/AI did not differ when EB and EC were compared at the end of the protocol (Melo et al., 2016). Thus, it seems likely that EC 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 insufficient circulating E2 concentrations from the combination of the EC treatment and the growing ovulatory follicle, which may produce a lack of GnRH/LH surge or an insufficient GnRH/LH surge to elicit ovulation (Sartori et al., 2001). Alternatively, small increases in P4 concentrations near the time of AI, due to lack of complete CL regression, could inhibit the E2-induced GnRH/LH surge and thereby prevent ovulation (Souza et al., 2007; Brusveen et al., 2009; Bisinotto et al., 2010; Giordano et al., 2012a, 2013). 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 EC treatment (Monteiro et al., 2015). Although we did not measure circulating P4 on the day of AI, P4 concentrations were unexpectedly greater ($P = 0.05$) in cows from the CHEM 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 EC is used as an ovulation inducer compared with 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 (Souza et al., 2007; Brusveen et al., 2009; Giordano et al., 2012a). Thus, if complete luteolysis has not occurred by the time of AI, ovulation to EC might be compromised because of the inhibition of the EC-induced GnRH-LH surge, as the action of E2 at the hypothalamus can be blocked by P4 (Robinson et al., 2000; Richter et al., 2002).

In our 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 3). In another study, cows were considered synchronized when they did not have a CL on the day of AI, but had a CL 7 d later (>90%; Pereira et al., 2014), which is very close to our finding that 87.3% of cows ovulated at the end of 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 (Monteiro et al., 2015; Melo et al., 2016). Using both criteria for synchronization, synchronized cows had greater ($P < 0.01$) fertility compared with cows not synchronized to the protocol (Table 3).

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 ovulations (Table 1 and Table 2). Multiple ovulations are responsible for the high undesired twinning rate in high-producing lactating dairy cows (Wiltbank et al., 2006). Several risk factors are related to multiple ovulations and might account for the high multiple ovulations in our study, such as high milk production (Fricke and 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 with nonovulating cows, also had elevated multiple ovulation (24.8%; Table 2). Although many risk factors may be involved in the occurrence of high multiple ovulations and twinning rate (Silvia del Rio et al., 2007), it is important to note that CL regression between d -10 and -3 in our study was greater than 50% (data not shown), probably due to the use of EB at the initiation of the protocol (Monteiro et al., 2015; Melo et al., 2016). This phenomenon could underlie, at least in part, the high incidence of multiple ovulation observed in the present study.

Pregnancy per AI on d 32 and 60 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 d 32 and 60 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 (Pancarci et al., 2002; Cerri et al., 2004; Galvão et al., 2004; Souza et al., 2007; Pereira et al., 2014, 2016). 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).

Finally, P/AI was greater ($P = 0.02$) in farm A compared with farm B, independent of treatments (Table 4). 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).

CONCLUSIONS

The use of P4 implants that produce different profiles of circulating P4 during the FTAI protocol did not affect follicular dynamics, synchronization rate, or P/AI. Thus, either autoclaving or chemical disinfection of a reused CIDR produced similar results in the type of FTAI protocol used in this study. Obviously, a limitation for interpreting this research in field situations is that no comparison was done with reused P4 implants in other FTAI protocols and no comparison was done with new P4 implants. Nevertheless, physiologically, 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 timing 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, cows that were correctly synchronized during the protocol were more likely to become pregnant to the FTAI protocol.

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Table 4. Fertility outcomes (LSM ± SEM) of lactating dairy cows on 2 farms that received reused 1.9-g intravaginal progesterone implants during a fixed time AI protocol that had been sanitized by autoclave (AUT) or chemical disinfection (CHEM)

Variable	Adjusted proportion ± SEM (no./no.)						P-value		
	Farm A			Farm B					
	AUT	CHEM		AUT	CHEM		Treatment	Farm	Treatment × Farm
Pregnancy/AI, % (no./no.)									
d 32	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
d 60	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, % (no./no.)	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

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