J. Dairy Sci. 107:5122–5131
https://doi.org/10.3168/jds.2023-23923

Comparison of gonadotropin-releasing hormone and estradiol benzoate plus gonadotropin-releasing hormone to initiate a progesterone-based timed artificial insemination resynchronization protocol in lactating dairy cows

Carlos E. C. Consentini,[1,2] Leonardo F. Melo,[3] Tattiany Abadia,[4] Bruno Gonzales,[5] Jessica C. L. Motta,[1] Rodrigo L. O. R. Alves,[1] Lucas O. e Silva,[1] Milo C. Wiltbank,[2] and Roberto Sartori[1]*

[1]Department of Animal Science, University of São Paulo, Piracicaba, SP 13418-900, Brazil
[2]Department of Animal and Dairy Sciences, University of Wisconsin–Madison, Madison, WI, 53706
[3]School of Veterinary and Animal Sciences, Federal University of Goiás, Goiânia, GO 74690-900, Brazil
[4]Céu Azul Farm, Silvânia, GO 75180-000, Brazil
[5]São Jorge Farm, São Pedro, SP 13520-000, Brazil

ABSTRACT

The present study compared 2 strategies to initiate a progesterone (P4)-based timed artificial insemination (TAI) protocol for lactating dairy cows: only GnRH or estradiol benzoate (EB) plus GnRH (EB+GnRH). Lactating Holstein cows (n = 487; 184 primiparous and 303 multiparous) from 2 commercial dairy herds were used for their second or greater services postpartum. Each week, cows that were nonpregnant at the pregnancy diagnosis 32 d after a previous AI were randomly assigned to 1 of 2 experimental groups that differed only in the strategy to initiate (d 0) the TAI protocol. On d 0, every cow received a 2.0-g P4 implant; in the EB+GnRH group, cows were treated with 2.0 mg i.m. of EB and 16.8 µg i.m. of the GnRH analog buserelin acetate, whereas in the GnRH group, cows received only 16.8 µg i.m. of GnRH. On d 7 after the initial treatment, 0.530 mg i.m. of cloprostenol sodium (PGF) was administered in all cows, followed by a second dose on d 8, concomitant with 1.0 mg i.m. of estradiol cypionate and P4 implant withdrawal. The TAI was performed on d 10 (48 h after P4 device withdrawal) in both experimental groups. Only conventional Holstein semen was used throughout the study. The percentage of cows with corpus luteum (CL) on d 0 (73%) and overall ovulation rate after d 0 (54%) did not differ between groups. The CL regression between d 0 and the first PGF treatment was greater in the EB+GnRH group than the GnRH group (42% vs. 31%). Consequently, the proportion of cows with CL at PGF was greater when only GnRH was used on d 0 compared with EB+GnRH (86% vs. 82%), and the mean number of CL at PGF was greater (1.23 vs. 1.11). The expression of estrus near TAI was greater in GnRH group (84% vs. 77%), and cows showing estrus had greater (44% vs. 10%) pregnancy per AI (P/AI) on d 32 for both treatments. We found no effect of the presence of CL on d 0 or at PGF, nor of ovulation after d 0 or CL regression between d 0 and d 7 on fertility. However, fertility was critically impaired when cows did not have CL at both times, d 0 and at PGF treatment. We did not observe any interaction between treatment and other variables, and the P/AI was similar in cows receiving EB+GnRH or only GnRH on d 0 (37.8% vs. 36.6%). In summary, although there was no detectable difference in P/AI between treatments, this study demonstrated potential negative physiological outcomes caused by EB treatment on d 0 (greater incidence of luteolysis after d 0 and fewer cows with CL at PGF treatment). Overall, we found no benefit of adding EB at the initiation of a P4-based TAI protocol on fertility compared with using GnRH alone, despite differences in ovarian dynamics and expression of estrus.

Key words: timed artificial insemination, estradiol benzoate, GnRH, fertility, dairy cow

INTRODUCTION

Timed artificial insemination (**TAI**) protocols have been widely used in dairy herds, thereby reducing the need for detection of estrus and improving the reproductive efficiency and profitability of dairy operations (Ricci et al., 2020; Consentini et al., 2021). To increase the 21-d pregnancy rate, cows that fail to become pregnant after an AI must be rapidly identified and re-inseminated. To shorten the interbreeding intervals, herds detect estrus for second and greater services, although the success of this strategy relies on the service rate, based on efficiency of the herd in detecting estrus. Alternatively, insemination of nonpregnant cows can be performed after resynchronization of ovulation using TAI protocols (commonly

Received June 30, 2023.
Accepted January 11, 2024.
*Corresponding author: robertosartori@usp.br

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

termed Resynch), usually initiated at the time of, or before, the nonpregnancy diagnosis (Lopes et al., 2013; Giordano et al., 2016; Wijma et al., 2017).

Usually, Resynch TAI protocols initiated at the nonpregnancy diagnosis are Ovsynch-type protocols (Lopes et al., 2013; Carvalho et al., 2015) or estradiol (**E2**) plus progesterone (**P4**)-based protocols, which are initiated with E2 benzoate (**EB**) and intravaginal P4 implants, sometimes combined with GnRH treatment (Pereira et al., 2015; Melo et al., 2018; Tschopp et al., 2022). Nevertheless, certain physiological aspects of the E2/P4-based protocols can impair fertility. For instance, treatment with EB was associated with ~40% corpus luteum (**CL**) regression between d 0 and the day of cloprostenol sodium (**PGF**) treatment (Melo et al., 2016). In addition, emergence of a new follicular wave was not properly synchronized in 25% to 40% of the cows (Monteiro et al., 2015; Melo et al., 2018), resulting in ovulation of a persistent follicle following TAI. Because lower circulating P4 during follicle development (Giordano et al., 2012a) and aged ovulatory follicles (Monteiro et al., 2015) affect fertility, both physiological situations are undesirable in lactating dairy cows.

Including GnRH at the initiation of the TAI protocol either without or with EB increased P/AI, compared with cows receiving only EB (Consentini et al., 2021). Nevertheless, many veterinarians and producers, especially from South America, continue to use EB combined with GnRH at the initiation due to a lack of studies comparing GnRH alone at the start of the protocol to GnRH plus EB. Thus, the present study compared these 2 strategies, evaluating the potential negative effect of EB treatment on physiological responses and fertility. Two main hypotheses were proposed for the study: (1) cows receiving EB on d 0 of the TAI protocol would have greater incidence of CL regression between d 0 and PGF treatment of the protocol, resulting in fewer cows with CL and a smaller number of CL at the time of PGF; and (2) cows initiating the protocol only with GnRH would have greater fertility than cows that received EB plus GnRH.

MATERIALS AND METHODS

This experiment was conducted in 2 commercial dairy farms, and the Animal Care and Use Committee of the Luiz de Queiroz College of Agriculture of the University of São Paulo (ESALQ/USP) approved all procedures involving cows in this study (protocol no. 2017.5.11620.11.3).

Animals, Housing, Herd Management, and Diets

In farm 1, the cows were housed in freestall barns with ventilation by fans, whereas farm 2 had cross-ventilation facilities. In both farms, cows were milked thrice a day, had free access to water and mineral salt, and were fed ad libitum with a TMR based on corn silage as the main forage and concentrate based on corn and soybean meal with minerals and vitamins balanced to meet or exceed the nutritional requirements (NRC, 2001) of lactating dairy cows producing approximately 40 kg/d of milk.

The reproductive management of both farms was 100% TAI for first service and a combination of estrus detection and TAI for re-insemination. Cows not detected in estrus and re-inseminated had their pregnancy diagnosis performed 28 to 34 d after AI, and those not pregnant were enrolled in the TAI protocols used in the experiment. In this study, cows in farm 1 (n = 260) were enrolled during the hot season of a tropical region (October–March), and farm 2 (n = 227) enrolled cows during an entire year. Lactating Holstein cows (n = 487; 184 primiparous and 303 multiparous) were used for their second (n = 158) or greater services (n = 327) postpartum. At the beginning of the experiment (d 0), cows were at 184.4 ± 102.8 DIM (± SD), with a BCS of 3.11 ± 0.03 (scale from 1 to 5 according to Ferguson et al., 1994).

Treatments and Experimental Design

Each week, nonpregnant cows at pregnancy diagnosis 32 d after a previous AI were randomly assigned according to parity and number of AI to 1 of 2 experimental groups (Figure 1) that differed only in the strategy to initiate (d 0) the TAI protocol. On d 0, every cow received a 2.0-g P4 implant (Repro sync, GlobalGen, Jaboticabal, Brazil). In the EB plus GnRH (**EB+GnRH**) group, cows were treated with 2.0 mg i.m. of EB (Syncrogen, GlobalGen) and 16.8 µg i.m. of the GnRH analog buserelin acetate (Maxrelin, GlobalGen), whereas in the GnRH group, cows received only 16.8 µg i.m. of GnRH. The higher dose of buserelin was chosen based on previous studies from our laboratory that have shown a decrease in LH peak and ovulation when a regular dose of GnRH was given in the presence of high circulating P4 (Motta et al., 2020; Silva et al., 2023, 2024). On d 7 after the initial treatment, 0.530 mg i.m. of cloprostenol sodium (**PGF**; Induscio, GlobalGen) was administered in all cows, followed by a second dose on d 8, concomitant with 1.0 mg i.m. of estradiol cypionate (**EC**; Cipion, GlobalGen) and P4 implant withdrawal. The TAI was performed on d 10 (48 h after P4 implant withdrawal) in both experimental groups. Within the protocols, all cows were inseminated at the scheduled time without changes due to expression of estrus at the end of the TAI protocol. Only conventional Holstein semen from multiple sires was used during the experimental period, only one technician in each farm performed the AI, and this technician was blind to the treatment groups.

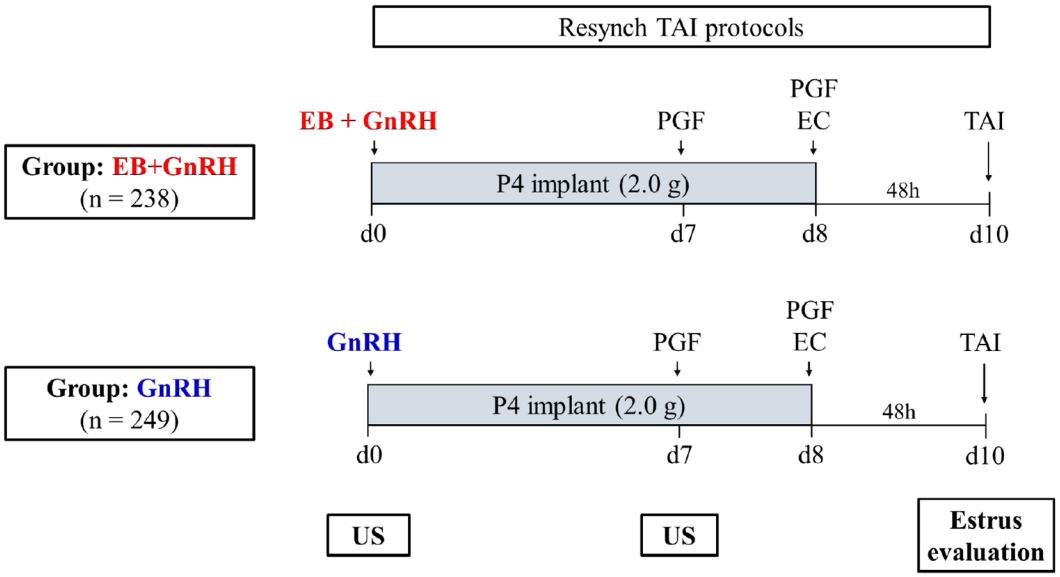

Figure 1. Experimental design with hormonal treatments and procedures performed during P4-based TAI protocols. On d 0, all nonpregnant cows received a 2.0-g P4 implant. In the EB+GnRH group, cows were treated with 2.0 mg of EB and 16.8 µg of GnRH (buserelin acetate), whereas in the GnRH group, cows received only 16.8 µg of GnRH. On d 7 after the initial treatment, 0.530 mg of PGF was administered in all cows, followed by a second dose on d 8, concomitant with 1.0 mg of EC and P4 implant withdrawal. The TAI was performed on d 10 (48 h after P4 implant withdrawal) in both experimental groups. Ultrasound (US) evaluations to check the presence and number of CL were performed on d 0 and d 7.

Ovarian Structures, Expression of Estrus, and Pregnancy Diagnosis

To assess the presence (independent of its diameter) and number of CL, and to evaluate the response to hormonal treatments, ultrasound (US) evaluations were performed on d 0 and d 7 in all cows. Ovulation was defined as cows that had a follicle greater than 8 mm on d 0 and, subsequently, a new CL was observed on d 7 (time of PGF treatment), similar to Pereira et al. (2015). Moreover, if the cow had a CL with a fluid-filled cavity on d 7 in the same ovary that had a compact CL on d 0, it was also considered as ovulation. Regression of CL during the protocol (between d 0 and d 7) was determined based on the disappearance of any CL present on d 0. The examination was performed with transrectal ultrasonography of the reproductive tract using a 5 to 8 MHz multifrequency linear-array transducer (Ibex Lite, E.I. Medical Imaging, Loveland, CO). For follicle diameter, perpendicular measurements at maximum distances between 2 opposite borders were taken using the built-in calipers of the ultrasound machine. The diameter was determined as the mean of these 2 measures.

Expression of estrus was evaluated using a rump-mounted patch (BOViFLAG, Bovitime Animal Products LTD, Stellenbosch, South Africa) placed on d 8 (at the time of P4 implant withdrawal and EC administration), and cows were considered expressing estrus when the device was activated (e.g., >50% of the silver scratch-off layer was rubbed off, changing the patch color) by d 10 (time of AI). Evaluation of the expression of estrus was performed only on d 10.

Pregnancy per AI (**P/AI**) was determined 32 d (both farms) and 60 d (only on farm 1) after TAI by transrectal US of the reproductive tract by confirming the embryo heartbeat.

Statistical Analysis

The physiological measures were sufficiently powered to provide good reliability for detecting differences. However, there was some concern related to the possibility of a type II error for the binomial data, particularly for the P/AI results. A simple power calculation for a 10% difference (37% vs. 47%) using $\alpha = 0.05$ and a sample size per group of 238 gave a 0.6 power for a 2-tailed test and 0.72 for a one-tailed test. These conditions would result in sufficient power (≥0.80) to detect differences in P/AI of ≥13 percentage points (i.e., 36% vs. 49%).

Statistical analyses were performed using the Statistical Analysis System (SAS version 9.4 for Windows, SAS Institute Inc., Cary, NC). Analyses for continuous variables (number of CL at PGF treatment) were performed using the GLIMMIX procedure fitting a Gaussian distribution.

Analyses of binomial variables (presence of CL on d 0 and d 7, ovulation after d 0, CL regression after d 0, expression of estrus, P/AI on d 32 and d 60, and pregnancy loss) were performed using the GLIMMIX procedure

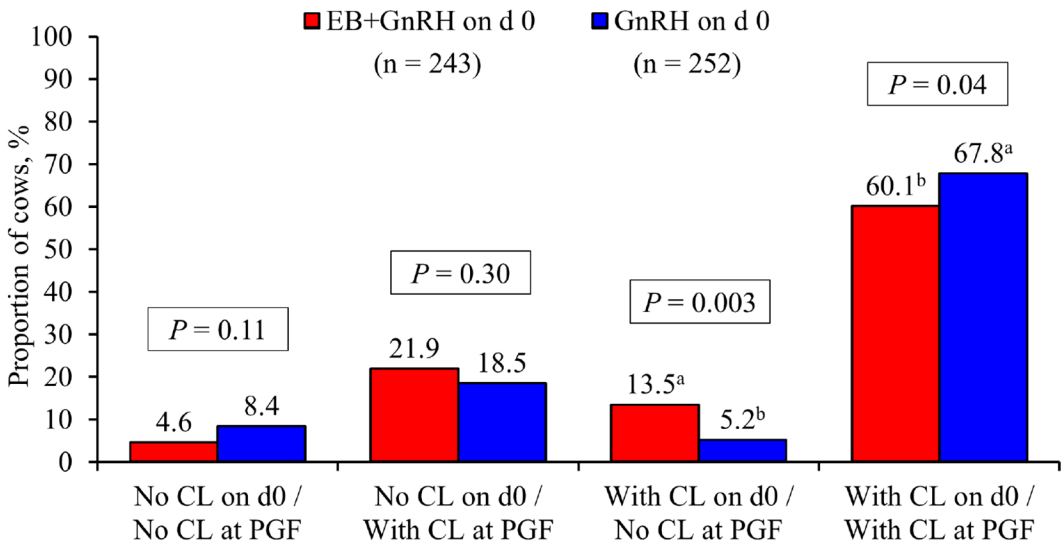

Figure 2. Proportion of cows with or without CL on d 0 and at the time of PGF treatment (d 7) based on the strategy to initiate the TAI protocol. Treatments were EB+GnRH or only GnRH on d 0. The dose of GnRH was 16.8 µg of buserelin acetate, and the dose of EB was 2 mg. [a,b]Different superscript letters indicate differences among experimental groups within the classes of cows.

fitting a binomial distribution with the Logit Link function. Additionally, the option ddfm = kenwardroger was included in the model statement to adjust the degrees of freedom for variances.

The selection of the model that best fit each variable of interest was performed by finding the model with the lowest value for the Akaike information criterion corrected using the *forward* procedure, removing variables with $P > 0.20$ from the model.

For the presence of CL on d 0, we studied the effects of treatment, farm, parity, AI number, and BCS on d 0 (≤2.5 or >2.5). The final model included the effect of treatment, farm, parity and BCS. Regarding ovulation after d 0, after studying the effects of treatment, farm, parity, BCS, AI number, and presence of CL on d 0, the final model included the effects of treatment, BCS and presence of CL. For CL regression during the protocol, the final model included the effect of treatment, after evaluating the effects of treatment, parity, farm, AI number, and interaction between treatment and parity. The final model for presence and number of CL on d 7 included the effects of treatment, farm, and BCS, although AI number, parity, and their interactions with treatment were also studied. A separate model was developed to evaluate the effect of ovulation and CL regression after d 0 on presence of CL on d 7. Regarding expression of estrus, the model included effects of treatment, farm, parity, BCS, and ovulation after d 0. The effects of CL presence on d 0 and d 7, CL regression during the protocol, and interaction between treatment and parity were also evaluated.

To better understand the effect of experimental treatments on CL dynamics during the protocol, 4 categories of cows were created (Figure 2): absence of CL on d 0 and d 7; absence of CL on d 0 and presence on d 7; presence of CL on d 0 and absence on d 7; and presence of CL on d 0 and d 7. Regarding classes of cows according to presence of CL on d 0 and d 7, the final model included the effects of treatment, farm, and parity, although BCS and interaction between treatment and parity were also studied.

The final model for P/AI on d 32 included the effects of treatment, BCS, parity, farm, AI number, CL on d 0, ovulation after d 0, and the interaction between CL and ovulation after d 0. Effects of the presence of CL on d 7 and interaction between treatment with parity, CL on d 0 and AI number were also studied. Separate models were built to evaluate the effects of expression of estrus, classes of cows according to CL on d 0 and d 7, and the interaction between ovulation and CL regression after d 0. The model for P/AI on d 60 only included data from farm 1 and included the effects of treatment and expression of estrus, and the model for pregnancy loss included only the treatment effect.

Tukey's honest significant difference post hoc test was performed to determine differences. Values are presented as means ± SEM for continuous variables and as a percentage for binomial variables. Significant differences were declared when $P \leq 0.05$, whereas tendencies were considered for $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

This research was conducted to evaluate both the physiological and fertility responses to only GnRH compared

with GnRH plus EB at the start of a synchronization of ovulation protocol. Because all cows were evaluated for some specific physiological endpoints, these responses had greater statistical power than many previous studies; however, the fertility responses may have been less powered than desired and subject to a type II error if a difference of less than 12% is present for P/AI.

Effect of Treatment on Ovarian Dynamics, Expression of Estrus, and Fertility

On d 0, the overall proportion of nonpregnant cows with CL detected by ultrasound was 73.3% (357/487, Table 1), similar to studies that reported about 30% of cows without CL at the time of pregnancy diagnosis (Bilby et al., 2013; Bruno et al., 2014; Santos et al., 2016). We did not observe an effect of EB treatment ($P = 0.91$) on the ovulatory response after d 0, and the overall outcome was 53.6% (261/487), as shown in Table 1, which was greater than the 30% to 40% achieved in other studies (Giordano et al., 2012a; Melo et al., 2016; Pereira et al., 2017). This result may be explained by the higher dosage of GnRH or the type of GnRH analog (i.e., buserelin acetate) used in our study. Other studies comparing doses of buserelin and gonadorelin reported that doubling the dosage of GnRH approximately doubled the peak of the LH surge (Giordano et al., 2012b), and that buserelin induced greater LH release than gonadorelin (Picard-Hagen et al., 2015; Silva et al., 2024).

Our hypothesis that more cows receiving EB would have luteolysis between d 0 and d 7 of the protocol was confirmed, as the EB+GnRH group had fewer cows with CL at the time of PGF and tended to have fewer CL at time of PGF (Table 1). Our data regarding luteolysis were similar to the observation of ~40% CL disappearance after EB treatment reported in previous studies (Monteiro et al., 2015; Melo et al., 2016). Nevertheless, both groups continued to have more than 80% of the cows with CL at PGF (Table 1), mostly due to the high ovulatory response (>50%) after GnRH treatment on d 0.

The treatments affected the proportion of cows within the 4 categories created according to the presence of CL on d 0 and at PGF treatment (Figure 2). Treatment only with GnRH on d 0 decreased the proportion of cows with CL on d 0 but no CL at PGF treatment, as well as increased the percentage of cows with CL at both time points (Figure 2). These results can be explained by the greater incidence of luteolysis between d 0 and d 7 in cows from the EB+GnRH group compared with only GnRH, with EB treatment decreasing the number of cows with CL at PGF. A previous study comparing EB and GnRH on d 0 of TAI protocols performed the same type of analysis and reported a greater percentage of cows with CL at the time of PGF treatment when GnRH was used compared with EB (77.3 vs. 58.3%; Melo et al., 2016).

The proportion (~80%) of cows expressing estrus near TAI in our study was similar to what was reported for TAI protocols using EC as an ovulation inducer (Pereira et al., 2015; Pereira et al., 2016; Consentini et al., 2019). Interestingly, the cows receiving only GnRH on d 0 had greater expression of estrus (Table 1). In the GnRH group, cows ovulating to the GnRH given on d 0 are expected to have follicular wave emergence earlier in the protocol, between 1 and 2 d after treatment (Melo et al., 2018). This should result in follicles with ~6 d of dominance at AI, thus providing adequate follicular size and E2 production to stimulate expression of estrus in most cows. Cows that did not ovulate after d 0 in the GnRH group could be at the beginning of a follicular wave, in which they would also have follicles with 6 or more days of dominance at the end of the protocol. Conversely, in the EB+GnRH group, cows that ovulate to the GnRH treatment would be expected to have similar follicular dynamics as the GnRH only group. However, the cows in the EB+GnRH group that did not ovulate to the GnRH are likely to have atresia of the follicles with later follicular wave emergence, between d 3 and 5 of the protocol (Souza et al., 2009; Monteiro et al., 2015). This would result in a younger and potentially smaller and less steroidogenic follicle at TAI, impairing the expres-

Table 1. Ovarian dynamics and expression of estrus according to the strategy for initiation of the TAI protocol

	Strategy to initiate the TAI protocol[1]		
Item	EB+GnRH	GnRH	*P*-value
Cows with CL on d 0, % (n/n)	73.5 (175/238)	73.1 (182/249)	0.98
Ovulation after d 0, % (n/n)	53.8 (128/238)	53.4 (133/249)	0.91
CL regression after d 0, % (n/n)	42.3 (74/175)	31.3 (57/182)	0.03
Cows with CL at PGF, % (n/n)	81.9 (195/238)	86.4 (215/249)	0.05
Number of CL at PGF, n (n)	1.11 (238)	1.23 (249)	0.07
Cows with 2 or more CL, % (n/n)	16.8 (40/238)	22.1 (55/249)	0.14
Expression of estrus, % (n/n)	76.9 (183/238)	83.5 (208/249)	0.04

[1]Treatments were EB+GnRH or only GnRH on d 0 of progesterone-based TAI protocols. The dose of GnRH was 16.8 µg of buserelin acetate and the dose of EB was 2 mg.

Table 2. Ovulation after d 0 of TAI protocols initiated with GnRH or EB+GnRH according to farm, parity, BCS, and presence of CL on d 0

Item	Ovulation after d 0, % (n/n)	*P*-value
Farm		
1	52.7 (137/260)	0.31
2	54.6 (124/227)	
Parity		
Primiparous	51.6 (95/184)	0.90
Multiparous	54.8 (166/303)	
BCS		
≤2.5	45.3 (43/95)	0.03
>2.5	55.6 (218/392)	
Presence of CL on d 0		
No CL	75.4 (98/130)	<0.001
With CL	45.7 (163/357)	

sion of estrus (Bello et al., 2006; Souza et al., 2007). This can be observed in the small but significant difference in expression of estrus (76.9% vs. 83.5%) between the treatment groups.

Ovulation to the GnRH treatment on d 0 (Table 2) was not affected by farm or parity, with both farms and both primiparous and multiparous cows having ~53% ovulation. We observed an effect of BCS, with cows with BCS ≤2.5 having 10.3 percentage points lower ovulation than cows with BCS >2.5. No interaction between BCS and treatment was observed ($P > 0.05$). The presence of CL had the greatest effect on ovulation to the d 0 GnRH, with a reduction of ~30 percentage points in cows that ovulated (Table 2). No interaction between presence of CL on d 0 and treatment was observed ($P > 0.05$). It was expected that cows with CL would have a lower ovulation because it is established that the presence of CL or high circulating P4 concentrations (or both) at the time of GnRH treatment decreases LH peak and ovulatory response (Giordano et al., 2012b; Stevenson and Pulley, 2016; Silva et al., 2023).

The rationale for the experimental design used in this study was based on previous results from the scientific literature and studies performed in our laboratory. When TAI protocols that were initiated with EB or with EB plus GnRH were compared, it was reported that the inclusion of GnRH improved fertility of lactating dairy cows, especially in those without CL (Pereira et al., 2015). In addition, a study from our laboratory compared EB and GnRH on d 0 and found that GnRH treatment produced a better P4 milieu during follicle development and tended to have greater fertility (Melo et al., 2016). Interestingly, both studies used gonadorelin and reported low ovulatory responses after GnRH treatment (36%), but still observed better physiological and fertility results when GnRH was used at the beginning of the TAI protocols. A recent compilation of studies comparing only EB with the use of GnRH to initiate TAI protocols (with or without EB) showed lower percentage of CL at PGF treatment and reduced fertility when only EB was used at the beginning (Consentini et al., 2021). Nevertheless, to our knowledge, no previous study has evaluated the effects of combining the EB treatment with GnRH compared with GnRH alone at the beginning of the TAI protocol. One limitation of our study was the use of a 7-d interval between ultrasound examinations to determine ovulation and CL regression, which could potentially confound the interpretation of our results.

The P/AI on d 32 after TAI was similar between treatments (Table 3), with no interaction between treatment and parity (Table 3). Considering only farm 1, the EB+GnRH and GnRH groups produced similar P/AI on d 32 after TAI (35.1% [46/131] vs. 31.8% [41/129]; $P = 0.56$) and d 60 after TAI (29.0% [38/131] vs. 28.7% [37/129]; $P = 0.91$) and we found no effect of treatment on pregnancy loss between d 32 and d 60 after TAI (17.4% [8/46] vs. 9.8% [4/41]; $P = 0.31$).

Thus, our hypothesis that cows receiving EB on d 0 would have lower fertility was not confirmed. As mentioned, the EB+GnRH group had greater incidence of luteolysis during the protocol and fewer cows with CL at the time of PGF than cows receiving only GnRH (Table 1). Nevertheless, the fertility was not lower. The lack of a negative effect of EB on d 0 on fertility could be partially explained by the relatively high ovulation obtained in this study (53%), which resulted in more than 80% of cows with CL at the time of PGF, even in the EB+GnRH group (Table 1). Other researchers have reported that inclusion of GnRH on d 0 of a TAI protocol initiated with EB in-

Table 3. Pregnancy per AI on d 32 after AI according to the strategy to initiate the TAI protocol

	Strategy to initiate the protocol[1]		*P*-value[2]		
P/AI on d 32, % (n/n)	EB+GnRH	GnRH	T	P	T × P
All cows	37.8 (90/238)	36.6 (91/249)	0.86	—	—
Parity					
Primiparous	40.2 (37/92)	37.0 (34/92)	0.86	0.88	0.56
Multiparous	36.3 (53/146)	36.3 (57/157)			

[1]Treatments were EB+GnRH or only GnRH on d 0 of TAI protocols. The dose of GnRH was 16.8 µg of buserelin acetate, and the dose of EB was 2 mg.

[2]T = effect of treatment; P = effect of parity; T × P = interaction between treatment and parity.

creased circulating P4 concentrations and percentage of cows with CL at PGF compared with cows receiving only EB on d 0 (Pereira et al., 2015). In addition, cows that ovulated at the beginning of TAI protocols would have a CL present at PGF, increased circulating P4 during the protocol, and this could possibly improve fertility, particularly in cows with low circulating P4 or without CL at the onset of the protocol (Giordano et al., 2012a; Melo et al., 2018).

Another aspect that could reduce the negative effect of the CL regression induced by EB treatment is that all cows received a 2.0-g P4 implant during the protocol. It is reported that insertion of 2 P4 implants (1.38 g each) did not produce similar circulating P4 concentrations as a mature CL (Bisinotto et al., 2015a); however, P4 supplementation with intravaginal devices has been associated with increased fertility, primarily in cows without CL (Bisinotto et al., 2010, 2015a,b). Therefore, in the EB+GnRH group, those cows undergoing luteolysis during the protocol may have benefited from the use of a 2.0-g P4 implant in the present study.

Effect of Ovarian Dynamics and Expression of Estrus on Fertility

The effect of several variables on P/AI are shown in Table 4. We did not identify any interactions between treatments and these variables on P/AI (not shown). We also found no effect of the presence of CL on d 0 or on d 7 on P/AI and no effect of the number of CL on d 7 on P/AI (Table 4). It has been reported that presence of CL and circulating P4 concentration at the beginning of TAI protocols or at PGF are positively associated with fertility in lactating dairy cows submitted to both Ovsynch-type and E2 plus P4-based protocols (Giordano et al., 2012a; Melo et al., 2016; Pereira et al., 2017; Borchardt et al., 2020). In a previous study, cows with CL at the time of PGF had a P/AI of 40% (418/1,045) on d 30 compared with 24% (133/551) attained by cows without CL (Pereira et al., 2017). Similarly, the presence of an active CL or circulating P4 ≥ 1.0 ng/mL at the time of PGF resulted in greater fertility of resynchronized cows (37% vs. 8%; Giordano et al., 2012a). Two aspects of the present study may have contributed to the lack of effect of CL at PGF on fertility. First, almost all cows (84.2%; 410/487) had a CL at the time of PGF. Second, all cows in the study received a 2.0-g P4 device during the protocol. Both of these factors would have increased circulating P4 concentrations during development of the preovulatory follicle and made it less likely to detect differences in P/AI.

To better understand the impact of the presence of CL during the protocol on fertility, the P/AI was evaluated based on the 4 CL categories, created by the presence or absence of CL on d 0 and at PGF (Figure 3). Cows without CL at the beginning and at the time of PGF had the lowest P/AI on d 32 after TAI and it was 43% lower than cows in the other 3 groups which were not different from each other (38.2%; 174/455). This result reinforces the importance of high circulating P4 concentrations during follicular development on fertility of lactating dairy cows. Two other studies that performed similar analyses also reported lower fertility in cows without CL compared with cows with CL during the protocol, especially in those without CL at both the beginning and at PGF treatment (Melo et al., 2016; Pereira et al., 2017).

Table 4. Pregnancy per AI 32 d after TAI according to ovarian dynamics and expression of estrus

Item	P/AI on d 32 after TAI, % (n/n)	*P*-value
Presence of CL on d 0		
No CL	34.6 (45/130)	0.59
With CL	38.1 (136/357)	
Presence of CL at PGF		
No CL	32.5 (25/77)	0.21
With CL	38.1 (156/410)	
Number of CL at PGF		
1	39.6 (107/270)	0.27
≥2	35.0 (49/140)	
Ovulation after d 0		
No ovulation	36.3 (82/226)	0.95
With ovulation	37.9 (99/261)	
CL regression after d 0		
No CL regression	38.5 (87/226)	0.78
With CL regression	37.4 (49/131)	
Expression of estrus		
No estrus	10.4 (10/96)	<0.001
With estrus	43.7 (171/391)	

We found no interaction ($P > 0.10$) between ovulation after d 0 and treatment or parity on fertility. In addition, ovulation after d 0 did not influence P/AI (Table 4). These results contrast with data from a study that reported greater fertility in cows ovulating at the beginning of the TAI protocol (Pereira et al., 2017). To further explore this effect, results for P/AI were divided by cows that had or did not have a CL at the beginning of the protocol, and then by whether they ovulated to GnRH (Table 5). Ovulation increased P/AI by 77% in cows without CL at the beginning of the protocol but had no effect on P/AI in cows with a CL (Table 5). The positive effect of ovulation in cows without CL has been previously reported (Giordano et al., 2012a), and is likely related to better synchronization of the follicular wave and greater circulating P4 during the protocol in cows that ovulated (Melo et al., 2018).

Another intriguing result obtained in the present study was that luteolysis during the protocol (between d 0 and PGF treatment) did not impact fertility (37.4% [49/131] vs. 38.5% [87/226] for cows with and without luteolysis after d 0, respectively). This lack of effect could be due to an elevation in circulating P4 in most cows during the protocol due to high ovulation incidence after d 0,

Table 5. Pregnancy per AI (%, n/n) on d 32 after AI according to presence of CL on d 0 and ovulation after d 0

	Ovulation after d 0		
Presence of CL on d 0	No	Yes	*P*-value[1]
No CL on d 0	21.9 (7/32)	38.8 (38/98)	0.05
With CL on d 0	38.7 (75/194)	37.4 (61/163)	0.80

[1]A tendency was found for an interaction between the presence of CL an ovulation on d 0 ($P = 0.08$).

high proportion of cows with CL at the time of PGF, and the presence of the 2.0-g P4 implant in all cows. We expected that ovulation after d 0 might have improved fertility in cows undergoing lysis of the pre-existing CL during the protocol. However, there was no impact of ovulation after d 0 on P/AI of cows undergoing luteolysis (38.0% [27/71] vs. 36.7% [22/60]; $P = 0.82$). The lack of a positive effect of ovulation on d 0 in cows undergoing luteolysis during the protocol may be related to the low percentage of cows without a CL on d 7 (26%) and the presence of the P4 implant (Bisinotto et al., 2015b).

In our study, expression of estrus did not interact with experimental treatments, but dramatically affected fertility, with cows expressing estrus having 4.2 times greater P/AI (Table 4). It is reported that expression of estrus at the end of TAI protocols increases fertility (Pereira et al., 2016; Consentini et al., 2019) with a reported increase of 13 percentage points in P/AI at d 30 and d 60 after AI in 5,430 cows submitted to TAI protocols with EC as the ovulation inducer. In addition, it is likely that there was a lack of ovulation to the protocol in many of the cows that did not show estrus, particularly because they were given EC as the ovulation inducer.

Effect of Farm, BCS, and Number of AI on Fertility

We identified a farm effect on fertility, in which farm 2 achieved 8 percentage points greater P/AI (41.4% [94/227] vs. 33.5% [87/266]; $P = 0.03$). This difference might be expected because farm 2 had better overall management and better comfort for the cows. The use of only 2 farms with different management methods may be considered a limitation of the study, although we did not observe a treatment by farm interaction. In addition, it is well established that thinner cows have decreased fertility compared with cows with BCS >2.5 (Souza et al., 2009). Our results support the effect of BCS, with cows with a lower BCS (≤2.5) having 37% lower fertility than cows with BCS >2.5 (25.3% [24/95] vs. 40.1% [157/392]; $P = 0.008$). Similar to other studies (Lopes et al., 2013), cows receiving the second and third AI had greater fertility than those receiving ≥4 AI (41.1% [65/158]^{a}, 41.4% [36/87]^{a}, and 33.1% [80/242]^{b}, respectively, where different superscript letters indicate differences among number of services; $P = 0.05$).

CONCLUSIONS

In conclusion, the results of this study demonstrate that TAI protocols initiated with EB plus GnRH or with only GnRH produced similar fertility. Even though treatment with EB produced greater incidence of luteolysis between d 0 and d 7 of the protocol, resulting in fewer cows with CL at the time of PGF treatment, we found no detectable difference between treatments on P/AI. Thus, there seems to be no fertility benefit or detriment of adding EB at the initiation of a resynchronization, P4-based TAI protocol in lactating dairy cows compared with us-

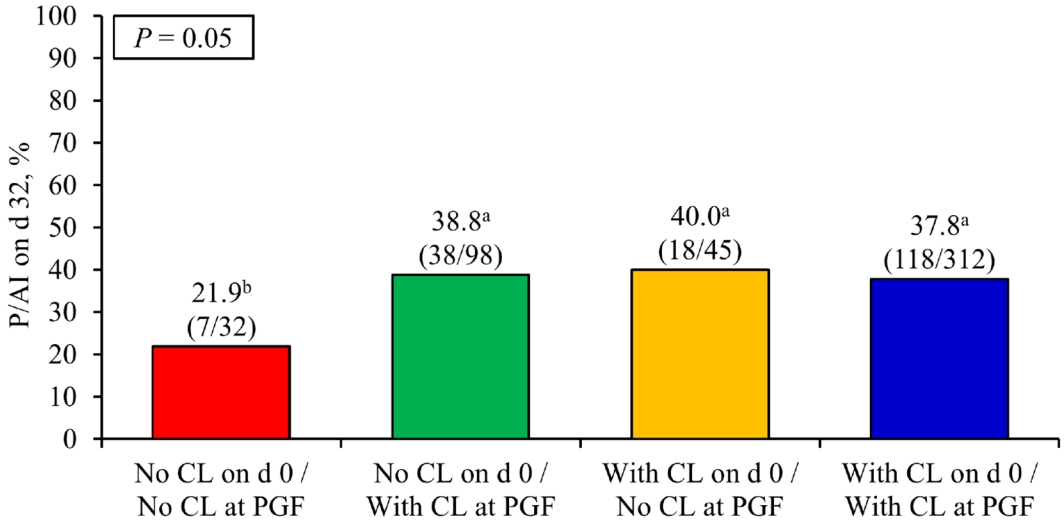

Figure 3. Pregnancy per AI 32 d after TAI in cows with or without CL on d 0 and at the time of PGF treatment of TAI protocols. [a,b]Different superscript letters indicate differences among experimental groups within the classes of cows.

ing GnRH alone, despite differences in ovarian dynamics and the expression of estrus.

NOTES

The authors thank the owners and staff of farms São Jorge (São Pedro, SP, Brazil) and Céu Azul (Silvânia, GO, Brazil) for the use of their cows and facilities. We appreciate the Coordination for the Improvement of Higher Education (CAPES, Brasília, Brazil) and Brazilian National Council for Scientific and Technological Development (CNPq, Brasília, Brazil) for the financial support for the experiment and students that worked during the experiment. We also thank the São Paulo Research Foundation (FAPESP, São Paulo, Brazil) for the support in the study by the scholarship grant no. 2017/15904-3 and fund grant no. 2018/03798-7. Our appreciation is extended to GlobalGen vet science (Jaboticabal, São Paulo, Brazil) for providing all hormones used in the TAI protocols. This experiment was conducted in 2 commercial dairy farms, and the Animal Care and Use Committee of the Luiz de Queiroz College of Agriculture of the University of São Paulo (ESALQ/USP) approved all procedures involving cows in this study (protocol no. 2017.5.11620.11.3). The authors have not stated any conflicts of interest.

Abbreviations used: CL = corpus luteum; E2 = estradiol; EB = estradiol benzoate; EB+GnRH = estradiol benzoate plus GnRH; EC = estradiol cypionate; P = effect of parity; P/AI = pregnancy per AI; P4 = progesterone; PGF = cloprostenol sodium; T = effect of treatment; T × P = interaction between treatment and parity; TAI = timed AI; US = ultrasound.

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