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Reproductive outcomes of lactating dairy cows submitted to first timed artificial insemination protocols with different strategies to induce final ovulation

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ABSTRACT

The study evaluated strategies for induction of ovulation at the end of timed AI (TAI) protocols initiated after a novel presynchronization strategy. A total of 909 lactating dairy cows from 6 dairy herds initiated a presynchronization protocol on d −15 with an intravaginal progesterone (P4) implant and 7 d later (d −8) were treated with 1.0 mg of estradiol cypionate (EC) and 0.530 mg of cloprostenol sodium (a synthetic analog of prostaglandin $F_{2\alpha}$; PGF), simultaneously with the P4 implant removal. On d 0, the protocol for synchronization of ovulation was initiated with 16.8 µg of buserelin acetate (a synthetic analog of GnRH) and insertion of a 2.0 g P4 implant, followed by a treatment with 0.530 mg of PGF on d 6, and a second PGF on d 7, concomitant with the P4 implant withdrawal. In Group EC, cows received 1.0 mg of EC on d 7 as an ovulation inducer. In G group, cows received 8.4 µg of GnRH 56 h after the first PGF (16 h before TAI). In Group EC/G, cows received both EC and GnRH. The TAI was performed on d 9 (48 h after P4 withdrawal) in all experimental treatments. There were no differences among treatments on pregnancy per AI (P/AI) on d 31 (40.4%; 367/909). There was a tendency for greater pregnancy loss in G group (19.8%) than in EC (12.2%) and EC/G (10.1%) groups. When the 2 groups that received EC were combined, the pregnancy loss was lower than when cows received only GnRH (11.2% [21/188] vs. 19.8% [17/86]; $P = 0.05$). There was an interaction between treatment and expression of estrus on P/AI, in which estrus positively affected P/AI in EC (53.3% vs. 20.0%) and EC/G (53.1% vs. 36.7%), but not in G group (41.5% vs. 38.7%). Overall, 81% of the cows had a corpus luteum (CL) on d 0 and 91% at PGF treatment of the breeding protocols. Fertility was greater in cows with CL at the time of the first PGF treatment than in cows without CL (45.9% vs. 17.7%). In summary, the strategies to induce final ovulation resulted in similar fertility despite the different physiological responses, such as expression of estrus, but further research is needed to definitively confirm the relationship between lack of EC and increased pregnancy loss.

Key words: timed AI, synchronization of ovulation, expression of estrus, fertility, dairy cow

INTRODUCTION

Reproductive efficiency directly affects profitability of dairy herds, and the focus of reproductive management strategies is to increase the number of cows becoming pregnant earlier in lactation. The use of timed artificial insemination (**TAI**) fertility programs, which include presynchronization strategies before the breeding protocols, optimize service rate by allowing prompt AI after the voluntary waiting period, and increases pregnancy per AI (**P/AI**) compared with reproductive management based on detection of estrus (Santos et al., 2017; Consentini et al., 2021; Fricke and Wiltbank, 2022).

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In this study, a novel estradiol (**E2**) and progesterone (**P4**)-based presynchronization strategy was used before GnRH- and P4-based TAI protocols aiming to increase the overall fertility, while strategies to induce ovulation at the end of the breeding protocol were compared. The presynchronization strategy aims to maintain a follicle growing during the period with a P4 device and to induce an ovulation after its removal and administration of cloprostenol sodium (a synthetic analog of prostaglandin $F_{2\alpha}$, **PGF**) and E2 cypionate (**EC**). Moreover, it fits in the weekly reproductive management calendar in commercial dairy farms.

Synchronized ovulation of the dominant follicle at the end of TAI protocols is an important aspect of TAI programs that can influence fertility. Final ovulation can be induced by E2 esters, such as E2 benzoate (**EB**) or EC (Melo et al., 2016) or with GnRH, as in Ovsynch-type protocols (Pursley et al., 1995). The use of EC is convenient because it can be administered either concomitant with the final PGF of the protocol or at the time of P4 implant removal, or at both times. However, the timing of ovulation induced by EC is more variable compared with when GnRH is used (Stevenson et al., 2004; Souza et al., 2009; Ferreira et al., 2017). In contrast, when GnRH is used at the end of TAI protocols, to achieve better fertility outcomes, cows need to be handled one more time, and the expression of estrus is reduced, which could compromise fertility, because lower expression of estrus and less circulating E2 concentration during the proestrus of TAI protocols have been associated with lower fertility and greater pregnancy loss (**PL**; Bello et al., 2006; Bisinotto et al. 2015a; Pereira et al., 2016).

The aim of the present study was to compare 3 strategies to induce/synchronize ovulation of the dominant follicle at the end of the TAI protocol using EC, GnRH, or both, and to evaluate the effects on reproductive outcomes such as expression of estrus, P/AI, and PL. Two main hypotheses were proposed: (1) cows with ovulation induced with GnRH and receiving E2 supplementation would have greater fertility than cows receiving only GnRH or only EC as ovulation inducers and (2) expression of estrus at the end of the TAI protocol would be greater in cows receiving EC, regardless of GnRH, and estrus would be associated with greater fertility.

MATERIALS AND METHODS

The experiment was conducted in 6 commercial dairy farms located in the southeast and midwest of Brazil. The Animal Care and Use Committee of Luiz de Queiroz College of Agriculture of University of São Paulo (ESALQ/USP) approved all procedures involving cows in this study (protocol # 2017.5.11620.11.3). Veterinarians, graduate students, and researchers were responsible for group allocations, hormonal treatments, BCS, estrus, and ultrasound (**US**) evaluations.

Animals, Housing, and Diets

The herds varied in size from 400 to more than 2,000 lactating cows, which were housed in freestall barns (farms 1, 2, 3 and 4) and cross-ventilated facilities (farms 5 and 6). All cows had free access to water and mineral salt and were fed ad libitum with a TMR diet balanced to meet or exceed the nutritional requirements of lactating dairy cows producing 40 kg of milk/d (NRC, 2001). Throughout the experiment, cows were milked thrice a day on all farms.

A total of 909 lactating Holstein cows were used for their first postpartum TAI. At the beginning of the experiment (d −15), cows were at 36.7 ± 0.3 DIM, yielding 38.9 ± 0.6 kg of milk/d, with BCS of 3.16 ± 0.02 (scale from 1 to 5, according to Ferguson et al., 1994). On d 0, milk production was 39.8 ± 0.7 kg of milk/d and BCS was 3.22 ± 0.02.

Treatments and Experimental Design

Weekly, a cohort of cows was submitted to a presynchronization protocol that was the same for all experimental groups. Presynchronization was initiated on d −15 with a 1.0-g P4 implant (Reproneo, GlobalGen, Jaboticabal, Brazil) previously used once, or with a 2.0-g P4 implant (Reprosync, GlobalGen) previously used once, twice, or thrice, and disinfected according to Melo et al. (2018). Seven d later (d −8), the P4 device was removed, and cows received 1.0 mg i.m. of EC (Cipion, GlobalGen) and 0.530 mg i.m. of cloprostenol sodium (Induscio, GlobalGen). Eight d after the end of the presynchronization protocol, a TAI protocol was initiated (d 0) and cows were randomly assigned, according to parity, to 1 of 3 experimental groups (Figure 1), that differed in the strategy to induce ovulation at the end of the protocol. On d 0, all cows received 16.8 µg i.m. of buserelin acetate (a synthetic GnRH; Maxrelin, GlobalGen) concomitant with the insertion of a 2.0-g P4 implant. On d 6, all cows received 0.530 mg i.m. of PGF followed by a second PGF injection on d 7, concomitant with P4 implant withdrawal. In **Group EC** (n = 318), cows received 1.0 mg i.m. of EC on d 7 as ovulation inducer. In **Group G** (n = 289), cows received 8.4 µg i.m. of GnRH on d 8.5 (56 h after first PGF and 16 h before TAI). In **Group EC/G** (n = 302), cows received EC on d 7 and GnRH on d 8.5. Cows were inseminated on d 9 (48 h after P4 implant withdrawal). The use of ~300 cows per group was decided because it would allow the detection of a 10% difference in P/AI (e.g., 40% vs. 50%, in EC or G vs. EC/G group, for instance) with reliability ($P = 0.05$) and a power of 0.77

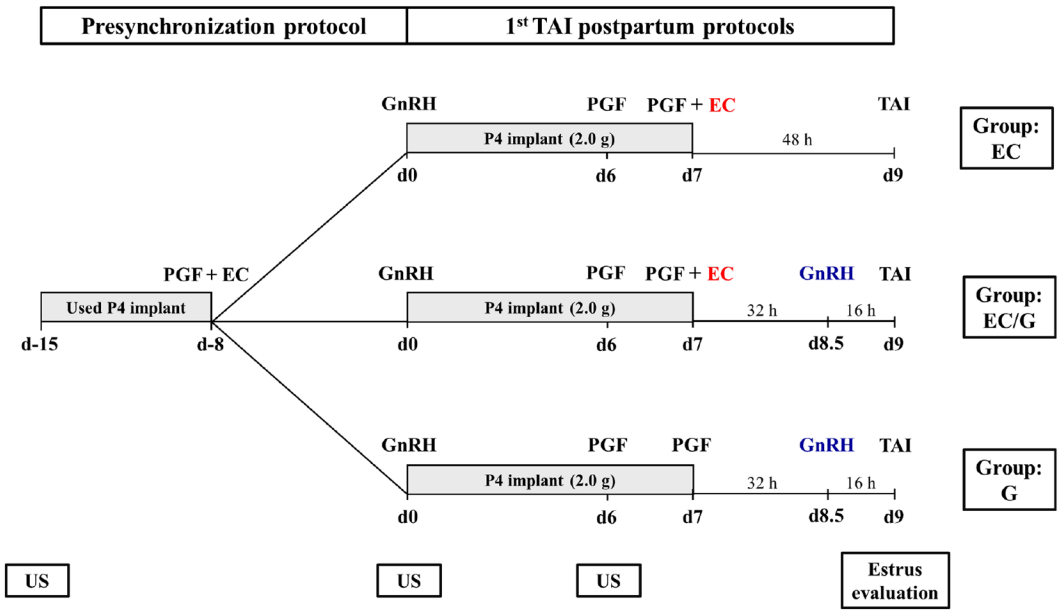

Figure 1. Experimental design with hormonal treatments and procedures performed during the presynchronization protocol and TAI protocols. Presynchronization protocol: On d −15, all cows received a 1.0-g (previously used once) or a 2.0-g (previously used once, twice, or thrice) P4 implant. Seven days later (d −8), the P4 implant was removed and 1.0 mg of estradiol cypionate (EC) and 0.530 mg of cloprostenol sodium (PGF) were administered. For TAI protocols, on d 0, cows from all groups received 16.8 µg of buserelin acetate (GnRH) and a 2.0-g P4 implant. On d 6, the first PGF treatment (0.530 mg) was administered, followed by a second dose on d 7, concomitant with the P4 implant withdrawal. All cows were inseminated on d 9 (48 h after the P4 implant removal). Group EC: Cows received 1.0 mg of EC on d 7. Group EC/G: Cows received 1.0 mg of EC on d 7 and 8.4 µg of GnRH on d 8.5. Group G: Cows received 8.4 µg of GnRH on d 8.5. Ultrasound (US) evaluations were performed on d −15, d 0, and d 6.

for a one-tailed test, using the PROC POWER package of SAS 9.4 (SAS Institute Inc., Cary, NC).

BCS, US Evaluations, Expression of Estrus, and Pregnancy Diagnosis

The BCS of cows was evaluated on d −15 and d 0 of the experimental design. To check for presence (independent of its size) and number of corpora lutea (**CL**) and to evaluate the response to hormonal treatments throughout the TAI programs, US evaluations were performed on a subset of cows on d −15 (n = 631), d 0 (n = 535), and d 6 (n = 362). Ovulation after d 0 was considered based on the appearance of a new CL on d 6 that was not present on d 0 and if the cow had a CL with a cavity on d 6 in the same ovary that had a compact CL on d 0. The examinations were performed by transrectal US of the reproductive tract using an 8 to 5 MHz multifrequency linear-array transducer (Ibex Lite, E.I. Medical Imaging, Loveland, CO).

A subset of cows (n = 368) received a tail-head device for detection of estrus (BOViFLAG, Bovitime Animal Products LTD, Stellenbosch, South Africa) on d 7, and the expression of estrus was considered when the device was activated (e.g., > 50% of the silver scratch-off layer was rubbed off, changing the patch color). The evaluation of expression of estrus was performed only at the time of AI (d 9).

Pregnancy diagnosis was performed 31 d after AI by transrectal US of the reproductive tract, confirming the embryo heartbeat. Pregnancy was confirmed 60 d after AI on farms 3, 4, 5, and 6, and PL between first and second diagnosis was calculated.

Statistical Analyses

Statistical analyses were performed using SAS (version 9.4 for Windows; SAS Institute Inc., Cary, NC). Analyses of binomial variables (presence of CL on d −15, d 0, and d 6, ovulation after d 0, expression of estrus, P/AI on d 31 and d 60, and PL) were performed using the GLIMMIX procedure fitting a binomial distribution with the Link Logit 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* selection procedure, removing variables with $P > 0.20$ from the model.

For presence of CL on d −15, d 0, and d 6, and ovulation after d 0, the final model included the effects of

treatment, farm, and BCS. The final model for expression of estrus included effects of treatment, farm, parity, and BCS. The interaction between treatment and parity and BCS was studied, as well as the effect of presence of CL on d 0 and d 6, although they had no effect.

For P/AI, the model included effects of treatment, farm, parity, and BCS. Presence of CL throughout the TAI program was evaluated, as well as ovulation after d 0. Interactions of interest were evaluated, and they are discussed in this manuscript, such as treatment with farm, parity, BCS, and presence of CL during the TAI protocol. A separate analysis was performed to evaluate the relationship between estrus and treatment on fertility, and BCS and treatment on expression of estrus and fertility. To independently evaluate the effect of estrus and BCS within treatment or the other way around, the SLICE command was used in the GLIMMIX procedure. For PL, the final model included the effect of treatment and farm, and a specific analysis was performed to compare both groups including EC combined with GnRH alone at the end of the breeding protocol.

The LOGISTIC procedure was used for regression to model the probability of pregnancy on d 31 according to BCS. Logistic regression curves were created using the coefficients provided by the interactive data analysis from SAS and the formula $Y = \exp(\alpha \times X + \beta)/[1 + \exp(\alpha \times X + \beta)]$, where Y = probability of occurrence; exp = exponential; α = slope of the logistic equation; β = intercept of the logistic equation; and X = analyzed outcome.

Values are presented as means ± SEM for continuous variables or as percentage for binomial variables. Significant differences were declared when $P \leq 0.05$, and tendencies were considered when $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Maximizing fertility to TAI programs requires the proper timing of AI in relation to ovulation, and an optimized hormonal environment to provide adequate gamete transport, fertilization, and embryo development (Dransfield et al., 1998; Dalton et al., 2001; Roelofs et al., 2006). This study included a novel presynchronization strategy that was similar to programs recently reported (Pereira et al., 2020; Consentini et al., 2021), focused on producing an adequate P4 milieu during development of the preovulatory follicular wave, which has been previously found to increase fertility during Ovsynch-type protocols (Wiltbank and Pursley, 2014; Santos et al., 2017; Carvalho et al., 2018). This design allowed a valid experimental evaluation of different strategies to induce final ovulation, because adequate synchronization and hormonal environment for the final follicular wave would be expected.

Effect of Strategy to Induce Final Ovulation on Fertility

Other studies have compared E2 vs. GnRH to induce ovulation or E2 supplementation (Pancarci et al., 2002; Stevenson et al., 2004; Sellars et al., 2006; Souza et al., 2007, 2009; Hillegass et al., 2008; Brusveen et al., 2009; Ferreira et al., 2017), although they differed from our experiment in several aspects. All previous experiments used only a single PGF treatment, although 2 PGF treatments are needed for complete CL regression in all cows and ensure optimal fertility (Wiltbank et al., 2015; Barletta et al., 2018), as used in our study. Our study used GnRH treatment at 16 h before AI, a more optimal time for fertility (Pursley et al., 1998) than the Cosynch (GnRH at same time as AI) strategy (Hillegass et al., 2008; Ferreira et al., 2017) or AI 24 h after GnRH treatment (Pancarci et al., 2002; Stevenson et al., 2004; Sellars et al., 2006) used in previous studies. In addition, treatment with EC at the time of the second PGF treatment and P4 implant withdrawal should synchronize timing of E2 increase with a rapid decrease in P4, potentially optimizing timing of estrus and ovulation compared with administration of PGF at time of high P4 (Souza et al., 2009; Ferreira et al., 2017) or simultaneous with GnRH (Sellars et al., 2006). Finally, there are studies that administered E2 8 h before (Souza et al., 2007) or at the time of GnRH given 16 h before TAI (Brusveen et al., 2009). Thus, to establish a fair comparison of ovulation inducers and fertility, experimental treatments were not chosen to optimize labor but to administer both treatments at what would be considered the most ideal times within the treatment protocol, to allow a valid comparison between different methods for induction of ovulation.

Our hypothesis that the EC/G group would achieve the highest P/AI, due to an expected better-synchronized ovulation in response to GnRH plus a greater expression of estrus due to E2 supplementation, was not completely supported. Instead, considering all herds used in the study, experimental groups had similar ($P = 0.76$) P/AI on d 31 (Table 1), with an overall P/AI of 40.4% (367/909). Previous studies comparing EC to GnRH as ovulation inducers also reported no differences in fertility (Pancarci et al., 2002; Souza et al., 2009). In addition, previous studies that supplemented EC into protocols that also used GnRH to induce ovulation also reported no differences in fertility (Sellars et al., 2006; Hillegass et al., 2008; Ferreira et al., 2017), as observed in our study.

Considering data from farms that performed pregnancy diagnosis on d 60, there was no difference in P/AI on both d 31 ($P = 0.87$) and d 60 ($P = 0.58$) among experimental treatments (Table 1). When all experimental groups were compared, PL tended to differ ($P = 0.10$;

Table 1. Pregnancy per artificial insemination (P/AI) at 31 and 60 d after TAI and pregnancy loss according to the strategy used to induce final ovulation

	Strategy to induce final ovulation[1]			
Item	EC	EC/G	G	*P*-value
P/AI on d 31[2]	41.8 (133/318)	40.4 (122/302)	38.8 (112/289)	0.76
P/AI on d 31[3]	42.5 (99/233)	43.0 (95/221)	42.8 (89/208)	0.87
P/AI on d 60[3]	37.1 (86/232)	37.5 (81/216)	33.7 (69/205)	0.58
Pregnancy loss[3,4]	12.2 (12/98)[B]	10.0 (9/90)[B]	19.8 (17/86)[A]	0.10

[A,B]Different letters indicate a tendency for differences among groups ($P = 0.10$).

[1]Values given as % (n/n).

[2]Data from all farms of the experiment.

[3]Data from farms 3, 4, 5, and 6, which provided information on 31-d and 60-d pregnancy diagnosis.

[4]When the 2 groups receiving EC (EC and EC/G) were combined, pregnancy loss was lower compared with group G ($P = 0.05$).

Table 1). Moreover, when the 2 groups that received EC (EC and EC/G) were combined, the PL was lower compared with cows receiving only GnRH (11.2% [21/188] vs. 19.8% [17/86]; $P = 0.05$). The greater PL in group G could be explained, physiologically, by a lower circulating E2 concentrations during proestrus and lower expression of estrus in the cows receiving only GnRH to induce final ovulation compared with cows receiving EC. As expected, previous studies have confirmed that treatment with EC increases circulating E2 and expression of estrus (Sellars et al., 2006; Hillegass et al., 2008). In addition, some previous studies have also reported greater PL in cows that do not express estrus compared with cows expressing estrus (Bisinotto et al., 2013; Pereira et al., 2016; Consentini et al., 2023).

In our study, supplementation with EC increased expression of estrus but did not alter P/AI, similar to other studies that supplemented E2 in protocols that also treated with GnRH to induce final ovulation. For example, Brusveen et al. (2009) reported greater expression of estrus but no improvement in fertility when cows received 0.5 mg of estradiol-17β at the time of the second GnRH treatment of Ovsynch. Similarly, in cows submitted to a Presynch-Ovsynch program, treatment with 1.0 mg EC 24 h after the PGF of Cosynch-48 or Cosynch-72 increased expression of estrus but had no effect on P/AI or on PL (Hillegass et al., 2008). Moreover, in the Presynch-Ovsynch-56 program, 1.0 mg estradiol-17β given 8 h before the second GnRH did not increase P/AI and did not reduce PL, despite the greater expression of estrus in cows supplemented with E2 (Souza et al., 2007).

When Souza et al. (2007) treated cows with 1.0 mg 17β estradiol 8 h before the final GnRH of Ovsynch, there was a positive effect of E2 supplementation on P/AI of primiparous cows. In our study, E2 supplementation did not improve fertility of any category submitted to the Ovsynch-type protocol (Table 2). We speculated that E2 supplementation could benefit multiparous cows due to their greater milk production and lower expression of estrus. However, we could not detect an interaction ($P = 0.63$) between parity and treatment on P/AI (Table 2). In addition, when we combined the groups that included EC, there was also no effect on fertility of multiparous ($P = 0.20$) and primiparous cows ($P = 0.94$). Regarding P/AI on d 60 of multiparous cows, there was no effect detected of EC groups compared with only GnRH ($P = 0.15$). Nevertheless, primiparous cows had much greater P/AI (60% increase in relative P/AI) than multiparous cows, although PL was not affected by parity (Table 2). Most studies report greater fertility in primiparous cows, particularly at the first postpartum TAI (Carvalho et al., 2014a; Giordano et al., 2016), perhaps related to lower milk production and fewer health problems, such as subclinical hypocalcemia (Reinhardt et al., 2011), and other metabolic or clinical disorders (Cheong et al., 2011; Pascottini et al., 2017). Another aspect that affects fertility of primiparous and multiparous cows is the size of the uterus. Baez et al. (2016), using Double-Ovsynch, reported greater P/AI on d 32 and 67 for primiparous compared with multiparous cows and the authors reported a larger uterine size for multiparous cows and observed a negative effect on fertility as size of the uterus increased, particularly in older cows.

Therefore, even with an optimized experimental design for comparing EC or GnRH as ovulation inducers and the potential positive combination of both, we did not detect differences among experimental groups in P/AI. However, our results suggest a potential positive effect of EC administered at the end of TAI protocols on reproductive efficiency of high-producing dairy cows, by reducing PL.

BCS and Fertility

At first service, greater loss of postpartum BCS and lower BCS near TAI are reported to compromise fertility (Carvalho et al., 2014a; Barletta et al., 2017). Therefore,

Table 2. Pregnancy per artificial insemination (P/AI) at 31 and 60 d after TAI and pregnancy loss according to the strategy to induce final ovulation and parity

	Strategy to induce final ovulation[1]			*P*-value[2]		
Item	EC	EC/G	G	T	V	T × V
P/AI on d 31[3]						
Primiparous	52.6 (61/116)	54.4 (56/103)	53.7 (51/95)	0.62	<0.001	0.63
Multiparous	34.3 (46/134)	36.9 (48/130)	28.8 (36/125)			
P/AI on d 31[4]						
Primiparous	54.8 (51/93)	57.5 (50/87)	61.0 (47/77)	0.88	<0.001	0.53
Multiparous	34.2 (40/117)	37.3 (41/110)	30.6 (33/108)			
P/AI on d 60[4]						
Primiparous	48.4 (45/93)	52.9 (45/85)	48.7(37/76)	0.58	<0.001	0.78
Multiparous	29.3 (34/116)	29.9 (32/107)	23.6 (25/106)			
Pregnancy loss[4]						
Primiparous	11.8 (6/51)	6.3 (3/48)	19.6 (9/46)	0.14	0.51	0.73
Multiparous	12.8 (5/39)	15.8 (6/38)	19.4 (6/31)			

[1]Values are given as % (n/n).

[2]T = treatment effect; V = variable effect; T × V = treatment × variable interaction.

[3]Data from all farms of the study.

[4]Data from farms 3, 4, 5, and 6, which provided information on 31-d and 60-d pregnancy diagnosis.

we explored the interaction of BCS with the strategy to induce final ovulation on expression of estrus and P/AI (Table 3 and Figure 2). First, regardless of BCS, cows in G group had lower expression of estrus than cows in EC or EC/G, and this was particularly apparent in thin cows in the G group. Second, regarding P/AI, in cows with BCS greater than 2.75, there was no treatment effect ($P = 0.82$); however, cows with lower BCS had decreased P/AI when only EC or G were used to induce ovulation compared with the combined, EC/G group (Table 3). Furthermore, there was a linear effect of increasing BCS on P/AI in the EC ($P = 0.02$) and G ($P = 0.0003$) groups, but not in the EC/G ($P = 0.16$) group (Figure 2). Similarly, Souza et al. (2007) reported that E2 supplementation increased expression of estrus and fertility in cows with BCS ≤ 2.50 but not ≥ 2.75.

Thus, cows with lower BCS benefit from receiving both the EC, which increases expression of estrus, and GnRH, which likely increases percentage of thin cows that ovulate at the end of the protocol. In contrast, any of the strategies for inducing final ovulation are adequate in cows with better BCS. This result is somewhat consistent with our first hypothesis, but with the caveat that it is only supported in cows with lower BCS and not in cows with adequate BCS.

Expression of Estrus and Fertility

As expected, the expression of estrus was greater in groups EC and EC/G than in G group (84.3% [107/127], 76.2% [96/126], and 46.1% [53/115], respectively; $P <$ 0.001). The 80.2% (203/253) of cows in estrus from EC

Table 3. Interaction of BCS with treatment (ovulation inducer) on expression of estrus and P/AI on d 31 in a TAI protocol[1]

		Strategy to induce final ovulation			
Item	Overall	EC	EC/G	G	*P*-value[2]
Expression of estrus					
BCS ≤2.75	60.8 (62/102)	81.6 (31/38)[a]	71.9 (23/32)[a]	25.0 (8/32)[b]	<0.001
BCS >2.75	73.2 (180/246)	85.7 (72/84)[a]	77.0 (67/87)[a]	54.7 (41/75)[b]	<0.001
P-value[3]	<0.001	0.52	0.75	0.01	
P/AI on d 31					
BCS ≤2.75	30.7 (61/99)	30.3 (20/66)[b]	38.1 (24/63)[a]	24.3 (17/70)[b]	0.05
BCS >2.75	44.0 (243/552)	44.8 (90/201)[a]	43.6 (81/186)[a]	43.6 (72/165)[a]	0.82
P-value[3]	<0.001	0.03	0.91	0.05	

[a,b]Different letters within rows indicate differences ($P \leq 0.05$) among experimental groups within each class of BCS.

[1]Values are given as % (n/n).

[2]The *P*-values in the rows indicate differences between BCS classes in overall P/AI and within each experimental group.

[3]The P-values indicate differences (P ≤ 0.05) between BCS classes in overall P/AI and within each experimental group.

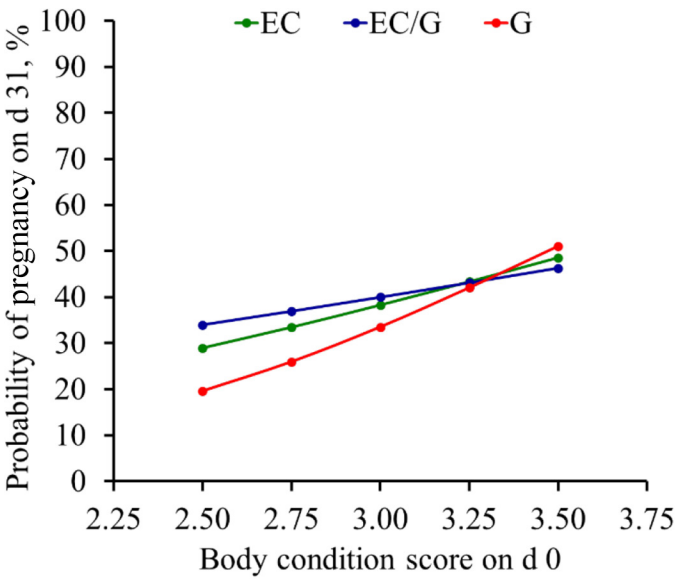

Figure 2. Probability curves for P/AI on d 31 according to BCS on d 0 of timed AI protocols with different strategies to induce final ovulation. Linear effect: group EC ($P = 0.02$), group EC/G ($P = 0.16$), and group G ($P = 0.0003$).

and EC/G groups was similar to previous studies that used EC at the time of P4 device withdrawal. For instance, Pereira et al. (2015) reported 83.2% of 1,780 cows expressed estrus with ovulation induced by EC. Related to the EC/G group, previous studies also reported greater expression of estrus in cows receiving E2 supplementation when their final ovulation was induced by GnRH compared with cows receiving only GnRH (Souza et al., 2007; Brusveen et al., 2009). Last, expression of estrus in cows from the G group (46.1% [53/115]) was higher compared with other studies that used GnRH to induce ovulation reporting 24% to 35% estrus (Ribeiro et al., 2012; Bisinotto et al., 2013, 2015a,b), but our results are similar to the 44% expression of estrus reported by Souza et al. (2007).

Parity influenced ($P = 0.002$) expression of estrus, with first- and second-lactation cows expressing more estrus (76.3% [184/241]) than cows in third or more lactations (58.1% [61/105]). This result was expected because older cows had greater milk production and would be expected to have lower circulating E2 due to increased steroid catabolism by the liver (Sangsritavong et al., 2002; Sartori et al., 2004).

In our study, there was a main effect of expression of estrus increasing fertility (50.8% [130/256] vs. 34.8% [39/112]; $P = 0.005$) and an interaction ($P = 0.03$) between estrus expression and treatment on P/AI on d 31 (Figure 3), with cows in G group having no benefit of estrus, cows in EC/G having some benefit, and cows in EC group having the greatest increase in P/AI with expression of estrus (20.0% [4/20] vs. 53.3% [57/107]; $P = 0.006$). Combining results for all cows receiving GnRH (Groups EC/G and G), the expression of estrus tended to increase fertility (38.0% [35/92] vs. 49.0% [73/149)]; $P = 0.09$). Previous results have shown that expression of estrus at the end of TAI protocols increased P/AI by ~10% on d 32 (50.2% vs. 40.4%) and on d 60 (46.0% vs. 35.8%) for cows submitted to Ovsynch-type protocols (Bisinotto et al., 2015b), although another study from the same group (Bisinotto et al., 2010) reported no effect on P/AI for cows expressing estrus at TAI, at either the pregnancy diagnosis on d 32 (45.6% vs. 46.2%, respectively) or d 60 (39.6% vs. 39.9%). Similarly, in the Presynch-Ovsynch-56 program, there was no effect of expression of estrus on P/AI at 38 and 61 d after TAI (Souza et al., 2007). Similar to our results, Pancarci et al. (2002) found that expression of estrus influenced P/AI only in cows with final ovulation induced by EC and not in cows with ovulation induced by GnRH. Likewise, in TAI protocols using EC as the ovulation inducer, a retrospective study with 5,430 cows showed that expression of estrus increased P/AI on d 30 and d 60 by 13% and was associated with lower PL (Pereira et al., 2016).

Expression of estrus may have a more pronounced effect on cows treated with EC compared with those treated with GnRH. In EC-treated cows, the absence of estrus may indicate a failure to induce a GnRH/LH surge, and consequently, a failure to ovulate, which would significantly compromise fertility. Additionally, the lack of estrus could suggest a lack of follicular synchrony, particularly in the EC-treated group, potentially leading to issues with the timing of ovulation. Moreover, a subset of cows treated with EC may exhibit pharmacological estrus even with smaller preovulatory follicles, whereas

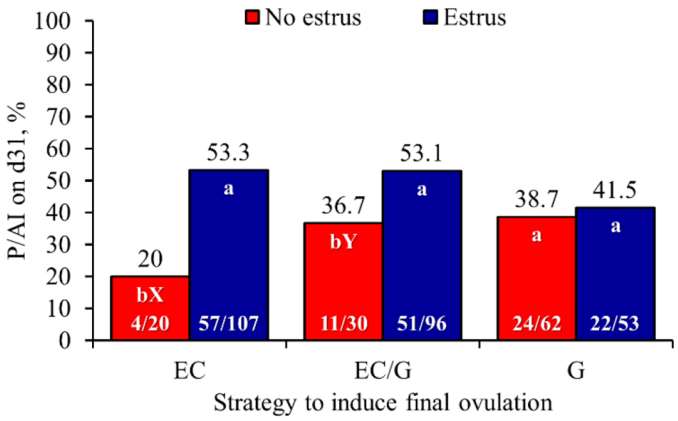

Figure 3. Pregnancy per artificial insemination (P/AI) 31 d after TAI according to strategy to induce final ovulation and expression of estrus. a,b: Different lowercase letters in the columns within the group represent differences ($P \leq 0.05$). X,Y: Different uppercase letters represent differences ($P = 0.10$).

in the GnRH-treated group, estrus is induced exclusively by endogenous E2. Treatment with GnRH, regardless of estrus, likely ensured that most cows ovulated near the time of AI, which may explain the absence of an estrus effect in Group G cows in our study.

Ovarian Dynamics

The proportion of cows without CL at the beginning of the presynchronization protocol (d −15; ~37 DIM) was not different ($P = 0.58$) among experimental groups (32.3% [204/631]) and similar to other studies in early lactation (Monteiro et al., 2021; Manríquez et al., 2021). The BCS affected cyclicity status on d −15 and d 0, with greater percentage of cows with CL when BCS was > 2.5 compared with thinner cows (Table 4). The proportion of cows with CL on d 0 ($P = 0.006$) and at PGF treatment ($P = 0.05$) was also affected by BCS (Table 4). Variation in BCS early in lactation can be related to many factors, including BCS at calving, clinical disease, length of the dry period, and the BCS itself and its loss during early lactation are associated with cyclicity resumption (Santos et al., 2009; Barletta et al., 2017; Monteiro et al., 2021). The lower CL presence on d 0 and at PGF of thinner cows indicated that BCS not only affected cyclicity but also ovulation to the presynchronization and to d 0. For example, cows with BCS > 2.5 had ~11 percentage points greater ovulation incidence than thinner cows (64.6% [193/299] vs. 53.7% [29/54]; $P = 0.10$).

There was no effect of parity on presence of CL on d −15, d 0, and d 6, and on ovulation rate after d 0. These results indicate that primiparous and multiparous cows had similar and good overall response to the TAI program, for instance, both had >90% of cows with CL present at PGF treatment (91.7% [155/169] and 90.3% [187/207], respectively).

The proportion of cows initiating the TAI protocols with CL was 80.9% (Table 4), which is similar to or greater than what has been reported in other reproductive programs that included presynchronization strategies. For instance, Ayres et al. (2013) and Dirandeh et al. (2015) reported 68% and 77%, respectively, of cows with CL at the onset of the Ovsynch protocol in the Presynch-Ovsynch program. Moreover, studies using Double-Ovsynch reported more than 90% of cows initiating the breeding Ovsynch with CL (Ayres et al., 2013; Carvalho et al., 2015).

Ovulation at the onset of Ovsynch protocols has been associated with greater P/AI (Giordano et al., 2013; Carvalho et al., 2015; Borchardt et al., 2020). It is known that early diestrus is the optimal time to initiate Ovsynch protocols to maximize the ovulatory response to GnRH (Vasconcelos et al., 1999), and in our study, overall ovulation incidence was 63.0% (Table 4). Ovulation in response to first GnRH was lower than from other studies that reported more than 80% ovulation to the first GnRH of the TAI protocol in Presynch-Ovsynch or Double-Ovsynch-synchronized cows (Gumen et al., 2012; Ayres et al., 2013). However, the ovulation rate of our study was similar to those of other studies that reported ovulatory responses of ~60%–70% (Souza et al., 2008; Giordano et al., 2013; Giordano et al., 2016).

The proportion of cows with CL at the time of the first PGF of the TAI protocols was high (~90%) and similar to studies that submitted cows to Presynch-Ovsynch or Double-Ovsynch (Ayres et al., 2013; Carvalho et al., 2015; Giordano et al., 2016). This is an exciting result because presence of CL and higher circulating P4 at the time of PGF treatment of breeding protocols is associated with improved fertility (Melo et al., 2016; Consentini et al., 2021).

Fertility According to Farm, CL Status, and Ovulation After d 0

The P/AI 31 d after TAI was greater ($P < 0.001$) in farm 6 compared with the others (57.9% [110/190] vs. 35.3% [48/136] vs. 32.4% [36/111] vs. 34.7% [34/98] vs. 34.8% [49/141] vs. 38.6% [90/233] for farms 6, 1, 2, 3, 4, and 5, respectively). The pregnancy diagnosis on d 60

Table 4. Proportion of lactating dairy cows with CL on d −15 (beginning of presynchronization protocol), d 0 (first GnRH of breeding protocols), and d 6 (first PGF of breeding protocols), and ovulation rate after d 0, according to BCS[1]

		Body condition score		
Item	Overall	≤2.5	>2.5	*P*-value
Presence of CL on d −15	67.7 (427/631)	52.9 (46/87)	69.9 (378/541)	0.004
Presence of CL on d 0	80.9 (433/535)	68.5 (50/73)	83.0 (375/452)	0.006
Presence of CL at PGF	91.0 (342/376)	83.6 (46/55)	92.0 (207/301)	0.05
Ovulation after d 0	63.0 (228/362)	53.7 (29/54)	64.6 (293/299)	0.10

[1]Values given as % (n/n).

was performed in farms 3, 4, 5, and 6, and likewise, farm 6 had the greatest (P = 0.002) P/AI on d 60 compared with the other 3 herds (49.5% [91/184] vs. 29.6% [29/98] vs. 25.9% [36/139] vs. 34.5% [80/232] for farms 6, 3, 4, and 5, respectively).

Regarding effects of the presence of CL on d −15, d 0, and d 6, as well as number of CL on d 6, there were no interactions of these variables with treatment, BCS, parity, ovulation after d 0, and estrus. For example, there was no statistically detectable effect of presence of CL on P/AI despite numerically greater P/AI on d 31 for cows with CL compared with those without CL on d −15 (44.7% [191/427] vs. 38.7% [79/204]; P = 0.48) or d 0 (44.3% [192/433] vs. 37.3% [38/102]; P = 0.37). It has been reported that cows that resumed cyclicity earlier in the postpartum period had increased fertility (Ribeiro et al., 2016) and cows initiating TAI protocols without CL or with low circulating P4 concentrations had lower P/AI (Herlihy et al., 2012; Ribeiro et al., 2012; Bisinotto et al., 2015a). It should be mentioned that the negative effect of absence of CL on d 0 on P/AI may have been reduced in our study because all cows received a 2.0-g P4 implant during the breeding protocols. Previously, Bisinotto et al. (2015a) reported that supplementation with two 1.38-g P4 implants in cows without CL at the beginning of the Ovsynch protocol produced similar fertility compared with cows initiating Ovsynch with CL. Only a few cows did not have CL present at the time of the first PGF treatment, with 91% having CL, although the P/AI on d 31 was greater for cows with CL (45.1% [148/328] vs. 17.7% [6/34]; P = 0.01). These results were expected, because the presence of CL on d 6 indicates that the ovulatory follicle has developed under higher circulating P4. Supporting this finding, other studies reported greater fertility in cows with CL compared with cows without CL or with low circulating P4 concentrations at the time of PGF treatment (Giordano et al., 2013; Carvalho et al., 2014b; Melo et al., 2016).

The presence of 2 CL at the time of PGF possibly indicates that one CL was formed after ovulation to the presynchronization, and the other was from ovulation of the dominant follicle at the time of the first GnRH of the breeding protocols. Indeed, fertility was usually greater in synchronized cows that had high P4, produced by 2 CL, at the time of PGF administration, as reported by Giordano et al. (2013) and Carvalho et al. (2014b, 2015). For our study, an additional analysis was performed based on the type of CL present at PGF. Cows were divided into 4 classes as shown in Figure 4: cows without CL, cows with only a CL from the presynchronization protocol, cows with only a CL from the ovulation to GnRH on d 0, and cows with 2 CL (one from presynchronization and another one from ovulation to d 0 GnRH). Cows with

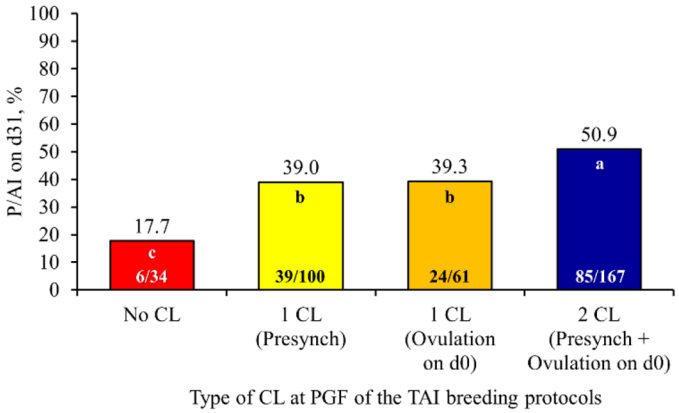

Figure 4. Pregnancy per artificial insemination (P/AI) 31 d after TAI based on number and type of CL at the time of the first PGF treatment of the breeding protocols. Cows were categorized into 4 classes: without CL at PGF, only a CL from the presynchronization protocol, only a CL by ovulation to GnRH on d 0, and cows with 2 CL (1 from presynchronization and another from ovulation to d 0 GnRH). a–c: Different letters indicate difference (P = 0.01).

2 CL achieved the greatest fertility (Figure 4), which could be due to the combination of a better P4 milieu during follicle development (Giordano et al., 2013; Melo et al., 2016), better synchronization of the preovulatory follicular wave emergence, and more optimal adequate age of the ovulatory follicle (Monteiro et al., 2015; Melo et al., 2018).

Although studies reported no effect of ovulation in response to the first GnRH of Ovsynch on fertility, especially in cows with high P4 at the beginning of the protocol (Giordano et al., 2016), generally cows that ovulate achieve greater P/AI on d 30 (Giordano et al., 2013; Bisinotto et al., 2015b; Carvalho et al., 2015) and d 60 (Chebel et al., 2006; Bisinotto et al., 2015b) after TAI. In our study, no interactions existed between treatment and ovulation on d 0 or presence of CL on d 0 on fertility but, overall, cows ovulating to GnRH on d 0 of the breeding protocol had greater P/AI on d 31 (47.8% [109/228] vs. 33.6% [45/134]; P = 0.04). As discussed previously, ovulation at the initiation of TAI protocols could improve fertility due to the increase in P4 and synchronization of the preovulatory follicular wave (Giordano et al., 2013; Monteiro et al., 2015).

CONCLUSIONS

This study has shown that the strategies to induce final ovulation in the TAI program produced similar overall fertility. In addition, the combination of EC and GnRH increased the fertility of cows with low BCS. Further research is needed to confirm if treatment with EC during the final ovulation induction protocol may reduce PL.

NOTES

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Nonstandard abbreviations used: CL = corpus luteum; E2 = estradiol; EB = E2 benzoate; EC = E2 cypionate; Group EC = cows received EC on d 7 as an ovulation inducer; Group EC/G = cows received both EC and GnRH; Group G = cows received GnRH 56 h after the first PGF (16 h before TAI); P4 = progesterone; P/AI = pregnancies per AI; PGF = prostaglandin $F_{2\alpha}$ (cloprostenol sodium is a synthetic analog); PL = pregnancy loss; T = treatment effect; TAI = timed AI; US = ultrasound; V = variable effect.

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