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Effect of progesterone from corpus luteum, intravaginal implant, or both on luteinizing hormone release, ovulatory response, and subsequent luteal development after gonadotropin-releasing hormone treatment in cows

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ABSTRACT

This study aimed to determine the effect of circulating progesterone (P4) concentrations produced by a corpus luteum (CL) or released by an intravaginal P4 implant (IPI) on GnRH-induced LH release, ovulatory response, and subsequent CL development, after treatment with 100 μg of gonadorelin acetate (GnRH challenge). Nonlactating multiparous Holstein cows were synchronized and GnRH was used to induce ovulation (d −7). Over 4 replicates, cows that ovulated (n = 87) were randomly assigned to a 2 × 2 factorial arrangement (presence or absence of CL and insertion or not of an IPI at GnRH challenge), creating 4 groups: CL_IPI, CL_NoIPI, NoCL_IPI, and NoCL_NoIPI. On d −1.5, NoCL_IPI and NoCL_NoIPI received 2 doses of 0.53 mg of cloprostenol sodium ($PGF_{2\alpha}$), 24 h apart to regress CL. On d 0, cows were treated with 100 μg of GnRH and, simultaneously, cows from IPI groups received a 2-g IPI maintained for the next 14 d. Diameter of dominant follicle, ovulatory response, and subsequent CL volume were assessed by ultrasonography on d −1.5, 0, 2, 7, and 14. Blood samples were collected on d −1.5, 0, 1, 2, 3, 5, 7, and 14 for analysis of circulating P4 and at 0, 1, 2, 4, and 6 h after GnRH challenge for analysis of circulating LH. In a subset of cows (n = 34), the development of the new CL was evaluated daily, from d 5 to 14. The presence of CL at the time of GnRH challenge affected the LH peak and ovulatory response (CL: 5.3 ng/mL and 58.1%; NoCL: 13.2 ng/mL and 95.5%, respectively). However, despite producing a rapid increase in circulating P4, IPI insertion did not affect LH concentration or ovulation. Regardless of group, ovulatory response was positively correlated with LH peak and negatively correlated with circulating P4 on d 0. Moreover, new CL development and function were negatively affected by the presence of CL and by the IPI insertion. In summary, circulating P4 produced by a CL exerted a suppressive effect on GnRH-induced LH release and subsequent ovulation of a 7-d-old dominant follicle, whereas the IPI insertion at the time of GnRH had no effect on LH concentration or ovulation. Finally, elevated circulating P4, either from CL or exogenously released by the IPI, compromised the development and function of the new CL, inducing short cycles in cows without CL at the time of GnRH treatment.

Key words: progesterone device, GnRH, gonadorelin, luteinizing hormone, ovulation

INTRODUCTION

Over the last 4 decades, several studies have developed diverse strategies to manipulate the estrous cycle and induce ovulation in cows by the administration of exogenous reproductive hormones (Knickerbocker et al., 1988; Bo et al., 1995; Sales et al., 2012; Wiltbank and Pursley, 2014). Analogues of GnRH have been widely used as inducers of ovulation, by binding to the pituitary GnRH receptor (**GnRHR**) and inducing release of LH (Clarke and Cummins, 1982; Peters, 2005; Souza et al., 2009a). During synchronization of ovulation protocols for timed-artificial insemination (**TAI**), such as Ovsynch, treatment with GnRH is used at both the beginning of the protocol, to synchronize emergence of the ovulatory follicular wave, and at the end, to synchronize final ovulation (Pursley et al., 1995; Souza et al., 2009b). Currently, GnRH-based TAI protocols are intensively used in reproductive management of dairy cows, improving both service rate and fertility (Carvalho et al., 2018; Vasconcelos et al., 2018; Consentini et al., 2021).

The efficacy of TAI protocols is associated with synchronized emergence of a new follicular wave at the beginning of the protocol (Monteiro et al., 2015), which leads to a satisfactory length of follicular dominance

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and ovulatory follicle age, improving fertility due to a better oocyte and embryo quality (Cerri et al., 2009b; Wiltbank et al., 2011). However, several studies evaluating reproductive strategies based on the Ovsynch protocol, starting at random stages of the estrous cycle of lactating dairy cows, reported only ~50% ovulation in response to the first GnRH of the protocol (Bilby et al., 2013; Bisinotto et al., 2013; Lopes et al., 2013).

Ovulatory response to GnRH treatment can be influenced by multiple factors, including stage of the estrous cycle (Vasconcelos et al., 1999), ovulatory capacity of the follicle (Sartori et al., 2001), chemistry of the GnRH analog (Souza et al., 2009a; Luchterhand et al., 2019), and circulating progesterone (**P4**) concentrations at the time of GnRH administration (Lima et al., 2013; Carvalho et al., 2015). Of particular interest, circulating steroid concentrations have dramatic effects on the magnitude of LH secretion in response to GnRH (Stevenson and Pulley, 2016; Motta et al., 2020). For example, Giordano et al. (2012a) compared the response to an i.m. treatment with 100 μg gonadorelin in cows with low circulating P4 (proestrus) or high circulating P4 (d 7 of cycle) concentrations, and reported a 5-fold greater LH peak in low compared with high P4 environment. Thus, circulating P4 modulates responsiveness of the pituitary gland to GnRH and, thereby, suppresses LH secretion (Nett et al., 2002).

In addition to synchronizing new follicular wave emergence, ovulation to the first GnRH leads to the formation of a new corpus luteum (**CL**), increasing circulating P4 during the development of the preovulatory follicle. For high-producing dairy cows, elevated P4 concentrations during follicular development increases oocyte and embryo quality, improving fertility (Rivera et al., 2011; Wiltbank et al., 2012). Thus, P4 supplementation by insertion of an intravaginal P4 implant (**IPI**) at the time of the first GnRH treatment has been frequently used to increase circulating P4 concentrations during TAI protocols, with positive results, particularly in cows initiating the protocol without CL (Chebel et al., 2010; Bisinotto et al., 2015). The magnitude of the increase in circulating P4 after insertion of an IPI (1.9 g P4) was found to be ~1 ng/mL (Melo et al., 2018) at 2 h after insertion in nonlactating Holstein cows and ~0.8 ng/mL (Cerri et al., 2009a) in lactating Holstein cows at 15 min after IPI insertion (1.38 g P4). Thus, there is increasing P4 concentration at the earliest stages of the GnRH-induced LH surge. Nevertheless, the effect of this rapid increase in P4 concentrations near GnRH treatment on GnRH-induced LH secretion and ovulatory response remains controversial. Previous studies reported around 35% reduction in ovulatory response for cows receiving an IPI at the time of GnRH (Galvão et al., 2004; Stevenson et al., 2008), although this effect was not observed in other studies (Bilby et al., 2013; Bisinotto et al., 2013).

Therefore, the objective of the present study was to determine the effect of circulating P4 concentrations produced by a 7-d-old CL, released by an IPI, or both, on GnRH-induced LH release, ovulatory response, and subsequent CL development, after treatment with a conventional dose of gonadorelin. Four main hypotheses were proposed: 1) circulating P4 originating from the CL would suppress the GnRH-induced LH surge; 2) insertion of an IPI at the time of GnRH treatment would rapidly increase circulating P4 concentrations and reduce a GnRH-induced LH surge; 3) the peak concentration of the LH surge, regardless of treatment group, would be associated with whether a cow ovulated after GnRH treatment; and 4) elevated circulating P4 concentrations at the time of GnRH treatment, especially due to the presence of a CL, would compromise the development and function of the new CL.

MATERIALS AND METHODS

The current study was conducted from October 2018 to March 2019 at the University of São Paulo (USP), Piracicaba, SP, Brazil. All procedures were previously approved by the Animal Research Ethics Committee of Luiz de Queiroz College of Agriculture (ESALQ/USP; Protocol CEUA # 2018–18).

Animals and Management

Nonlactating multiparous Holstein cows (n = 30; BCS = 3.3 ± 0.1) were kept in dry lots and were fed daily a maintenance diet based on haylage (*Cynodon dactylon*) and a corn plus soybean-based concentrate, with free access to water and mineral salt.

Experimental Design

Schematic representation of the experimental design is depicted in Figure 1. Initially, cows were submitted to a presynchronization protocol, starting on d −17 with the insertion of a 2-g IPI (Repro sync, GlobalGen vet science) that had been previously used for 7 d, concomitant with 2 mg estradiol (**E2**) benzoate i.m. (Syncrogen, GlobalGen vet science). Eight days later, on d −9, IPI were removed and all cows received 1 mg of E2 cypionate i.m. (Cipion, GlobalGen vet science), and 0.53 mg of cloprostenol sodium i.m. ($PGF_{2\alpha}$; Induscio, GlobalGen vet science). On d −7, 100 μg of gonadorelin acetate i.m. (GnRH; Fertagyl, MSD) was administered to induce ovulation. Cows were submitted to the experimental design over 4 replicates in this study. Exceptionally in the first replicate, only 24 cows

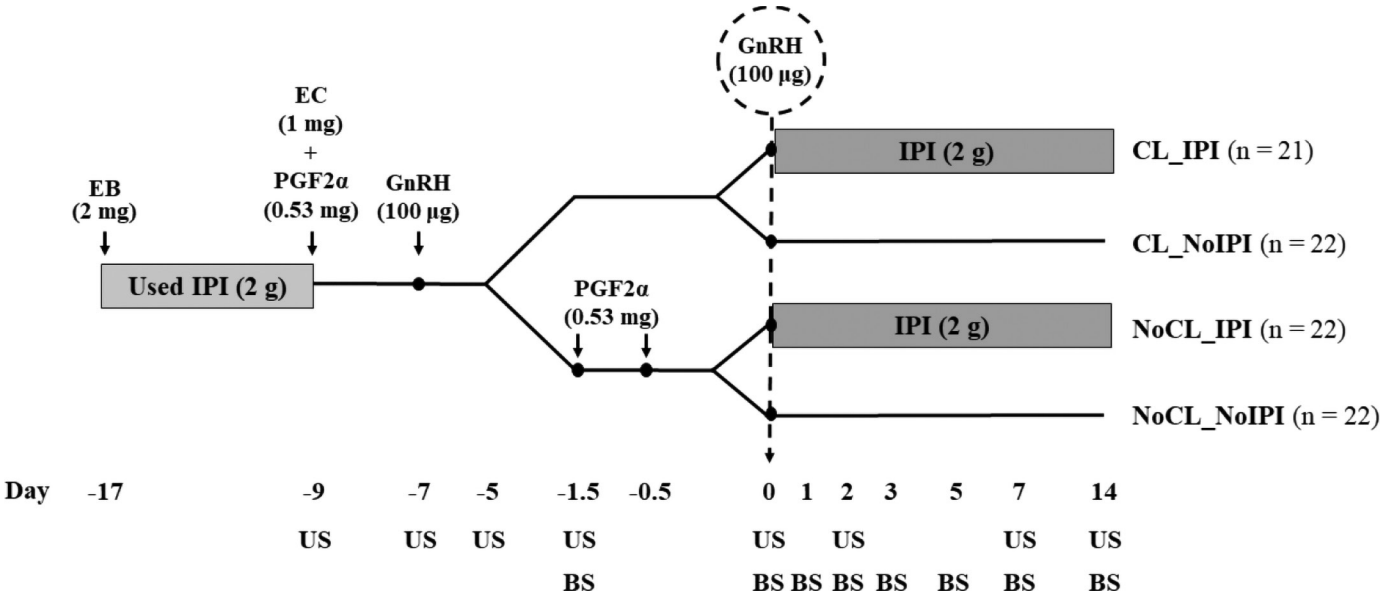

Figure 1. Schematic representation of the experimental design. Nonlactating Holstein cows were submitted to a presynchronization [d −17: estradiol benzoate (EB) + used intravaginal progesterone implant (IPI); d −9: IPI removal + estradiol cypionate (EC) + $PGF_{2\alpha}$; d −7: GnRH] to synchronize the follicular wave and generate a 7-d-old corpus luteum (CL). Four replicates were performed (R1: n = 24; R2: n = 30; R3: n = 30; R4: n = 30). Only cows that ovulated to the GnRH on d −7 and had a dominant follicle ≥10 mm on d 0 (n = 87) were randomly assigned to the experimental groups, with or without a CL on d 0 (CL vs. NoCL) combined with or without treatment with an IPI (2 g; Repro sync, GlobalGen vet science) on d 0 (IPI vs. NoIPI), inserted at the same time as GnRH treatment (100 µg; Fertagyl, MSD). Timing of $PGF_{2\alpha}$ treatments to regress original CL (d −1.5 and −0.5 in NoCL cows), GnRH treatment (d 0 in all cows), and IPI treatment (d 0 to 14 in IPI cows) is shown. Timing of ultrasound examinations (US) and blood samples (BS) is shown. In addition, on d 0, blood samples were collected at 0, 1, 2, 4, and 6 h from GnRH treatment (all cows). In a subset of cows (n = 34), ultrasound examinations and blood samplings were performed daily from d 5 to 14.

were submitted to the presynchronization protocol. From the second replicate, 6 more cows were included in the trials, to increase the total number of observations, considering possible losses in experimental units when cows were not properly synchronized. Over the 4 replicates, a total of 114 cows were submitted to the presynchronization protocol. Two cows lost the IPI during presynchronization and were removed from the replicate before d −7. Only cows that ovulated at the end of the presynchronization program, in response to the GnRH administration on d −7, and had a dominant follicle (**DF**) ≥10 mm on d 0 were submitted to the treatments (n = 90). Three observations were excluded because cows had a DF <10 mm on d 0. Then, 87 cows were randomly assigned to a 2 × 2 factorial arrangement composed by presence or absence of a 7-d-old CL and insertion or no insertion of a new IPI (2 g) at the time of GnRH challenge on d 0. Therefore, 4 groups were created by the combination of these factors: presence of CL and IPI insertion (**CL_IPI**; n = 21); presence of CL and no IPI insertion (**CL_NoIPI**; n = 22); no CL and IPI insertion (**NoCL_IPI**; n = 22); and no CL and no IPI insertion (**NoCL_NoIPI**; n = 22). Cows were assigned to a different experimental group in each replicate. Groups designed to have no CL at the time of GnRH challenge were treated twice with $PGF_{2\alpha}$ i.m. (0.53 mg), 24 h apart, on d −1.5 and −0.5, aiming to regress the original CL, formed after ovulation to the GnRH administered on d −7. Additionally, these cows received a tail-head adhesive patch for detection of estrus (BOViFLAG; Bovitime Animal Products Ltd.) on d −1.5 at the time of the first $PGF_{2\alpha}$ treatment, to check for eventual estrous behavior between d −1.5 and GnRH challenge, which would lead to the exclusion of the cow in that replicate. However, no cow was detected in estrus before the GnRH challenge. On d 0, all cows were treated with 100 µg of GnRH. Simultaneously, for groups designated to receive the IPI, the cows received a new 2 g P4 intravaginal implant, which remained for 14 d.

Ultrasound Evaluations

Transrectal ultrasound evaluations were performed using a 7.5 MHz linear-array transducer (DP-2200 VET, Mindray) and the ovarian structures (CL and follicles ≥5 mm) were mapped in all examinations. For all these structures, 2 measures were taken, at right angles, to obtain the maximum distances between 2 opposite borders, and the diameter was determined as the mean

of these 2 measures. On d −9, −7, and −5, ovaries of the cows were scanned to evaluate the DF dynamics in response to the presynchronization protocol, and to check the ovulatory response to GnRH treatment on d −7. On d −1.5 and 0, the location and diameters of the DF and original CL were evaluated, and 2 d later (d 2), the ovulatory response to the GnRH challenge on d 0 was assessed. Ovulation was determined by the disappearance of the DF from d 0 to 2 and confirmed by the presence of a new CL on d 7. The diameters of the original and new CL and any fluid-filled CL cavity were measured on d 7 and 14. Moreover, in a subset of cows (n = 34), the new CL development was evaluated daily from d 5 to 14. For the groups designated to keep the original CL (groups CL_IPI and CL_NoIPI), when ovulation to d 0 in the same ovary that the original CL was observed, the new CL was distinguished by the presence of a cavity (eventually) or by the position in the ovary, using the ovarian map. For the CL analyses, diameter measurements were used to calculate the volume of CL (cm^3) using the formula for calculation of the volume of a sphere ($V = 4/3\pi r^3$; r = diameter/2). For CL with cavity, the volume of the cavity was also calculated by its diameter value, and the cavity volume was subtracted from the total CL volume.

Blood Sampling and Hormone Assays

Blood samples were collected by puncture of the jugular vein into 9-mL heparinized evacuated tubes (Vacutainer, Becton Dickinson) and immediately placed on ice. To evaluate circulating P4 concentrations, samples were collected on d −1.5 (before $PGF_{2\alpha}$ treatment), d 0 (before IPI insertion), d 3, 5, 7, and 14, in all groups. Additionally, for groups that received the IPI (groups CL_IPI and NoCL_IPI), P4 concentrations were evaluated in blood samples collected at 1, 2, 4, and 6 h after the IPI insertion on d 0, and on d 1 and 2. In the subset of cows evaluated for CL development from d 5 to 14, samples were also collected daily during this period, to evaluate P4 concentrations. Moreover, to assess the LH concentration in response to the GnRH challenge, blood samples were collected on d 0, just before the GnRH treatment, and at 1, 2, 4, and 6 h later, in all groups.

After blood collection, tubes were centrifuged at 1,700 × *g* for 15 min at 4°C and plasma was stored at −20°C. Plasma P4 concentrations were determined using a solid-phase radioimmunoassay kit containing antibody-coated tubes and 125I-labeled P4 (ImmuChem Coated Tube P4 125 solid-phase radioimmunoassay Kit, MP Biomedicals), validated for bovine plasma as previously described (Melo et al., 2018). Sensitivity and intra- and interassay coefficients of variation (CV) were 0.02 ng/mL, 5.3%, and 8.4%, respectively. The LH concentration analyses were performed by solid-phase radioimmunoassay (Bolt and Rollins, 1983; Bolt et al., 1990), with some modifications (Ginther et al., 1999). Sensitivity and intra- and interassay CV for LH were 0.04 ng/mL, 7.8%, and 12.2%.

Statistical Analyses

The experiment was designed and analyzed as a 2 × 2 factorial arrangement. All statistical analyses were performed using the SAS computational software version 9.4 (version 9.4 for Windows, SAS Institute Inc.). Data were tested for normality of studentized residuals using the UNIVARIATE procedure, according to the Shapiro-Wilk test, and homogeneity of variances was evaluated by the Levene test, using the Hovtest and Welsh methods. When necessary, data were transformed to logarithm (CL volume, circulating P4 and LH concentrations), and outliers were removed. Nonparametric analysis was performed by the Wilcoxon and Kruskal-Wallis tests, using the NPAR1WAY procedure, to analyze time to LH peak, which did not normalize even after transformation.

Continuous data (CL volume, circulating P4 and LH concentrations, DF diameter and growth rate) were analyzed by the MIXED procedure and, for all the analyses, the final model included the fixed effects of presence of CL, IPI insertion and interaction between these factors (CL × IPI), fitting a Kenward-Roger method to calculate the denominator degrees of freedom to approximate the *F*-tests. Also, the replicate was considered as a random effect. For the analyses of the new CL volume on d 7 and 14, the DF diameter on d 0 was used as a covariate.

Circulating LH and P4 concentrations over time were analyzed as repeated measures by the MIXED procedure. The final model included the fixed effects of presence of CL, IPI insertion, time, and the respective interactions. A separate analysis was performed for circulating P4 from d 0 to 2 in groups that received the IPI, including only the presence of CL, time, and their interaction as fixed effects. Replicate was considered as a random effect and cow within treatment was the subject effect. The Kenward-Rogers method was used for the degrees of freedom calculation and, for each analysis, the appropriate covariance structure was selected, according to the smallest AICC value. Additionally, the area under the curve (**AUC**) of GnRH-induced LH release was calculated by the trapezoid method using the GraphPad Prism software (version 7.0) and analyzed by the MIXED procedure of SAS.

The ovulatory response was evaluated by the GLIMMIX procedure, fitting a binary distribution response

Table 1. Dynamics of the original corpus luteum (CL) and circulating progesterone (P4) concentrations in each experimental group from d −1.5 to 3 of the experimental design[1]

	CL		No CL		*P*-value[2]		
Item	IPI (n = 21)	No IPI (n = 22)	IPI (n = 22)	No IPI (n = 22)	CL	IPI	CL × IPI
Original CL							
Volume on d −1.5, cm^3	2.4 ± 0.2	2.6 ± 0.2	2.6 ± 0.2	2.7 ± 0.2	0.26	0.31	0.63
Volume on d 0, cm^3	3.9 ± 0.4	4.7 ± 0.3	1.5 ± 0.1	1.6 ± 0.7	<0.01	0.09	0.17
Volume on d 2, cm^3	5.5 ± 0.5	7.3 ± 0.6	0.5 ± 0.1	0.7 ± 0.2	<0.01	0.10	0.27
Volume change from d −1.5 to 2, %	+154 ± 33	+192 ± 17	−81 ± 5	−77 ± 4	<0.01	0.20	0.85
Structural luteolysis d −1.5 to 2,[3] % (n/n)	0.0 (0/21)	0.0 (0/22)	95.5 (20/22)	90.9 (21/22)	—	—	—
Circulating P4 concentration							
d −1.5, ng/mL	1.3 ± 0.2	1.3 ± 0.1	1.1 ± 0.2	1.4 ± 0.2	0.61	0.28	0.77
d 0, ng/mL	2.4 ± 0.3	2.1 ± 0.2	0.03 ± 0.0	0.02 ± 0.0	<0.01	0.58	0.65
d 3, ng/mL	5.4 ± 0.4	3.6 ± 0.2	2.1 ± 0.1	0.04 ± 0.0	<0.01	<0.01	0.38
Functional luteolysis on d 0,[4] % (n/n)	0.0 (0/21)	0.0 (0/22)	100.0 (22/22)	100.0 (22/22)	—	—	—

[1]Values presented as mean ± SEM or as percentage.

[2]Treatment effects: CL = presence of a 7-d-old CL at GnRH treatment; IPI = insertion of an intravaginal progesterone implant at GnRH treatment; CL × IPI = interaction between presence of CL and IPI insertion.

[3]Structural luteolysis defined as volume of the CL decreased >50% from d −1.5 to 2.

[4]Functional luteolysis defined as circulating P4 concentration <0.2 ng/mL on d 0.

and considering the presence of CL, IPI insertion, and their interaction as fixed effects. The incidences of structural and functional luteolysis were not statistically compared, and are presented only as descriptive data. In addition, the LOGISTIC procedure was used for logistic regression to evaluate the probability of ovulation after the GnRH challenge, as a function of circulating P4 concentrations on d 0 and amplitude of LH peak. The final model included the group effect and was selected by backward elimination, testing for linear and quadratic effects.

Finally, when interactions were detected, the SLICE command was used to study the effect of each factor within the other, and to evaluate the effect within each time in repeated measure analyses. The Tukey-Kramer post hoc mean separation test was performed to determine differences. Significant differences were declared when $P \leq 0.05$ and a tendency was defined when $0.05 < P \leq 0.10$. Continuous data are presented as means ± SEM and binomial data are presented as percentage (%; n/n).

RESULTS

Dynamics of the Original CL and Circulating P4 Concentrations

On d −1.5, the volume of the original CL (originated from ovulation after the first GnRH treatment on d −7) and circulating P4 concentrations were similar among groups (Table 1). As expected, $PGF_{2\alpha}$ treatments on d −1.5 and −0.5 efficiently regressed the CL in groups designated to have no CL on d 0. In groups in which the original CL was maintained, the CL volume increased 173 ± 19% from d −1.5 to 2, whereas in groups in which the original CL was regressed by $PGF_{2\alpha}$ treatments, the mean reduction in CL volume was 79 ± 3% (Table 1). Structural luteolysis was determined when the original CL had ≥50% reduction in volume from d −1.5 to 2. Following this criterion, no cows (0/43) from groups with CL had structural luteolysis by d 2, whereas 93.2% (41/44) of cows from groups without CL underwent structural luteolysis.

Likewise, $PGF_{2\alpha}$ treatments on d −1.5 and −0.5 efficiently promoted functional luteolysis, decreasing dramatically the circulating P4 concentrations in groups designated to have no CL. The mean circulating P4 before the GnRH challenge, on d 0, was greater in groups with CL than in groups without CL (2.2 ± 0.2 vs. 0.02 ± 0.01 ng/mL; $P < 0.01$). Moreover, all cows from groups without CL had undergone functional luteolysis (circulating P4 <0.2 ng/mL) by d 0 (Table 1). Although there were 3 cows from groups without CL that were not considered to have structural luteolysis by the criterion established in this study, they were kept in the analyses because they had circulating P4 <0.2 ng/mL on d 0.

In groups that received an IPI on d 0, circulating P4 concentrations significantly increased 1 h after IPI insertion (Figure 2). In the group CL_IPI, the IPI produced a 2-fold increase in circulating P4 concentrations 1 h after IPI insertion, maintaining the mean P4 concentration greater than 5 ng/mL up to 48 h. Similarly, in group NoCL_IPI, the IPI stimulated a rapid increase in circulating P4 concentrations, increasing around 1 ng/mL at the first hour and doubling at 24 h after IPI

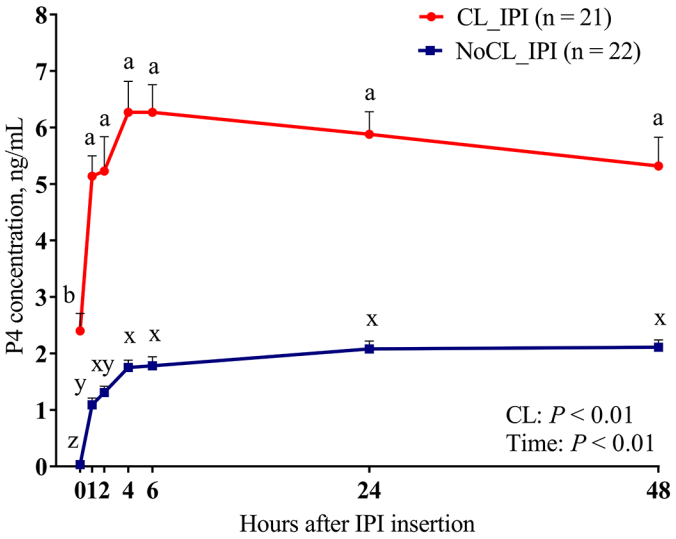

Figure 2. Circulating progesterone (P4) concentrations (mean ± SEM) from the time of intravaginal P4 implant (IPI) insertion (h 0), simultaneous with the GnRH treatment, to 48 h later, in cows with (CL_IPI) or without (NoCL_IPI) a 7-d-old corpus luteum. Different letters indicate differences over time within group CL_IPI (a, b; $P < 0.05$). Different letters indicate differences over time within group NoCL_IPI (x–z; $P < 0.05$).

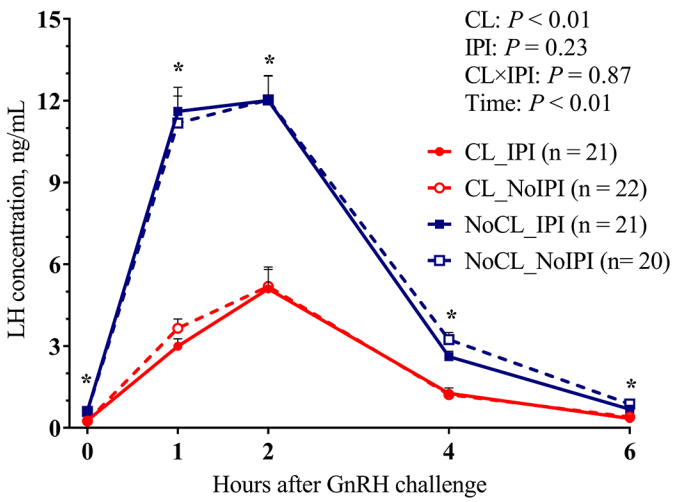

Figure 3. Circulating LH concentrations (mean ± SEM) from the treatment with 100 µg of gonadorelin (GnRH; h 0) to 6 h later, on d 0, based on the presence of a 7-d-old corpus luteum (CL), and the insertion of an intravaginal progesterone implant (IPI), at the time of GnRH challenge. Mean LH concentrations (ng/mL) at 0 h were: 0.3 ± 0.04, 0.3 ± 0.02, 0.6 ± 0.03, 0.6 ± 0.04; at 1 h: 3.0 ± 0.3, 3.7 ± 0.3, 11.6 ± 0.9, 11.2 ± 1.0; at 2 h: 5.1 ± 0.8, 5.2 ± 0.6, 12.0 ± 0.9, 12.0 ± 0.9; at 4 h: 1.3 ± 0.2, 1.2 ± 0.2, 2.6 ± 0.2, 3.2 ± 0.3; and at 6 h: 0.3 ± 0.04, 0.4 ± 0.04, 0.7 ± 0.05, 0.9 ± 0.05 in groups CL_IPI, CL_NoIPI, NoCL_IPI, and NoCL_NoIPI, respectively. Asterisks indicate the effect of presence of CL within each time ($P < 0.05$).

insertion (Figure 2). On d 3, as demonstrated in Table 1, there was a clear distinction in circulating P4 concentrations among groups, caused by the main effects of CL presence ($P < 0.01$) and IPI insertion ($P < 0.01$).

LH Release and Ovulatory Response

For the analyses of LH concentrations, data from 3 cows (NoCL_IPI: n = 1; NoCL_No IPI: n = 2) were considered outliers by evaluation of the studentized residuals and were excluded.

Despite promoting a rapid increase in circulating P4 concentrations, close to the time of the GnRH-induced LH surge, insertion of the IPI did not affect LH concentration ($P = 0.23$) and there was no CL × IPI interaction ($P = 0.87$) on circulating LH over time (Figure 3). Moreover, IPI insertion did not affect AUC ($P = 0.96$) or LH peak ($P = 0.82$; Table 2). Conversely, the presence of CL at the time of GnRH challenge negatively affected LH concentration over time ($P < 0.01$; Figure 3). Cows without CL had 2.5-fold greater circulating LH concentrations during the 6-h period after GnRH than cows with CL. The mean LH concentration (ng/mL) was greater in all observed times for groups without CL, but did not differ between groups with or without the IPI. Additionally, peak amplitude of LH surges after treatment with GnRH and AUC were greater ($P < 0.01$) in cows without CL than in cows with CL (13.2 ± 0.7 vs. 5.3 ± 0.5 ng/mL and 35.6 ± 1.5 vs. 14.2 ± 1.2 ng/mL*h, respectively). Mean time to LH peak was not affected by presence of CL ($P = 0.40$) or by IPI insertion ($P = 0.11$; Table 2).

Mean diameter of DF on d −1.5 was similar among groups (Table 3). However, there was a tendency ($P = 0.06$) for greater DF growth rate from d −1.5 to 0 for cows from groups without CL. Consequently, cows from these groups tended ($P = 0.10$) to have a larger DF on d 0 than cows with CL (Table 3). Nevertheless, no differences were observed in DF diameter on d 0 between cows that ovulated or did not ovulate to the GnRH challenge (12.4 ± 0.3 and 13.0 ± 0.2 mm, respectively; $P = 0.12$). As observed for the LH surges, presence of a CL at the GnRH challenge negatively affected the ovulatory response ($P < 0.01$), but there was no effect of IPI insertion ($P = 0.21$) or interaction between these factors ($P = 0.89$). Cows with a CL at the GnRH treatment had lower ovulatory response than cows without CL (58.1% vs. 95.5%; Table 3).

Regardless of treatment, cows that ovulated after the GnRH challenge had lower circulating P4 concentrations on d 0 than cows that did not ovulate (0.7 ± 0.1 vs. 2.5 ± 0.3 ng/mL; $P < 0.01$). This effect was also observed when cows from groups with CL were analyzed separately (1.8 ± 0.1 vs. 2.8 ± 0.3 ng/mL; $P = 0.01$). Figure 4 illustrates the distribution of cows that ovulated or not to the GnRH challenge, plotted in rela-

Table 2. Effect of presence of corpus luteum (CL), intravaginal progesterone implant (IPI) insertion, or both on GnRH-induced LH release[1]

	CL		No CL		*P*-value[2]		
Item	IPI (n = 21)	No IPI (n = 22)	IPI (n = 21)	No IPI (n = 20)	CL	IPI	CL × IPI
LH peak, ng/mL	5.3 ± 0.8	5.3 ± 0.6	13.4 ± 0.9	13.0 ± 1.0	<0.01	0.82	0.83
AUC,[3] ng/mL × h	14.0 ± 1.9	14.3 ± 1.5	35.8 ± 2.3	35.3 ± 2.0	<0.01	0.96	0.84
Time to LH peak, min	97.1 ± 6.5	106.4 ± 5.5	92.7 ± 6.5	98.2 ± 6.3	0.40	0.11	0.36

[1]Values presented as mean ± SEM.

[2]Treatment effects: CL = presence of a 7-d-old CL at GnRH treatment; IPI = insertion of an IPI at GnRH treatment; CL × IPI = interaction between presence of CL and IPI insertion.

[3]AUC = area under the curve.

tion to their circulating P4 concentrations on d 0 and the GnRH-induced LH peak. Most cows that did not ovulate had an LH peak <5 ng/mL. Moreover, the ovulatory response was linearly affected by circulating P4 concentrations on d 0 ($P < 0.01$), and by the amplitude of the GnRH-induced LH peak ($P < 0.01$). As circulating P4 increased, the probability of ovulation decreased (Figure 5A), whereas an increase in amplitude of the LH peak increased the ovulation risk (Figure 5B).

Development of the New CL and Circulating P4 Concentrations

Cows that ovulated to the GnRH challenge on d 0 (n = 67) had both the original (in groups with CL) and the new CL measured on d 7 and 14. On d 7, the IPI insertion tended ($P = 0.10$) to decrease the volume of the original CL; although, no difference was detected on d 14 ($P = 0.90$; Table 4). Regardless of the IPI insertion, in cows with CL at the GnRH treatment, the volume of the original CL decreased by ~60% from d 7 to 14. Moreover, 63.6% (7/11) of cows from group CL_IPI and 85.7% (12/14) of cows from group CL_NoIPI had the volume of the original CL reduced by ≥50% from d 7 to 14, i.e., structural luteolysis based on the same criterion previously presented (Table 4).

Regarding the volume of the new CL, 2 cows from the group NoCL_IPI were excluded from the analysis due to the presence of an extremely irregular cavity, precluding an accurate measurement. Moreover, unexpectedly, 1 cow from group NoCL_NoIPI had a very small new CL on d 7, which completely disappeared on d 14, and thereby, it was considered as an outlier and excluded from this analysis. For volume of the new CL on d 7, an interaction was detected ($P = 0.05$), whereby, in cows without the original CL, insertion of the IPI negatively affected the volume of the new CL ($P = 0.04$) whereas, in cows with the original CL, the IPI had no effect ($P = 0.52$). In addition, in cows that did not receive the IPI, the volume of the new CL was smaller when cows had the original CL ($P < 0.01$), whereas no effect of original CL was observed in cows that received the IPI ($P = 0.72$; Table 4). From d 7 to 14, except in cows from group NoCL_NoIPI, the mean volume of the new CL decreased substantially. Overall, in 60.1% (23/43) of cows from the other 3 groups, the volume of the new CL decreased ≥50% from d 7 to 14, indicating structural luteolysis. As a result, an interaction effect ($P < 0.01$) was detected on volume of the new CL on d 14, primarily related to the negative effect of IPI insertion on the volume of the new CL in the absence but not in the presence of the original CL ($P < 0.05$; Table 4). To better distinguish the effect of structural luteolysis on new CL volume, mean volume of CL on d 14 and volume change from d 7 to 14 are also presented separately for cows that underwent structural luteolysis or not, within each group. In addition, in 10 cows (group CL_NoIPI: n = 3; group NoCL_IPI:

Table 3. Dynamics of the dominant follicle (DF) before GnRH treatment and effect of presence of corpus luteum (CL), intravaginal progesterone implant (IPI) insertion, or both on ovulatory response[1]

	CL		No CL		*P*-value[2]		
Item	IPI (n = 21)	No IPI (n = 22)	IPI (n = 22)	No IPI (n = 22)	CL	IPI	CL × IPI
DF diameter on d −1.5, mm	10.5 ± 0.3	10.6 ± 0.3	10.7 ± 0.2	10.8 ± 0.4	0.50	0.75	0.95
DF diameter on d 0, mm	12.4 ± 0.3	12.8 ± 0.4	12.8 ± 0.3	13.5 ± 0.3	0.10	0.14	0.93
DF growth rate from d −1.5 to 0, mm/d	1.2 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	1.8 ± 0.2	0.06	0.15	0.39
Ovulation, % (n/n)	52.4 (11/21)	63.6 (14/22)	90.9 (20/22)	100 (22/22)	<0.01	0.21	0.89

[1]Values presented as mean ± SEM or as percentage.

[2]Treatment effects: CL = presence of a 7-d-old CL at GnRH treatment; IPI = insertion of an IPI at GnRH treatment; CL × IPI = interaction between presence of CL and IPI insertion.

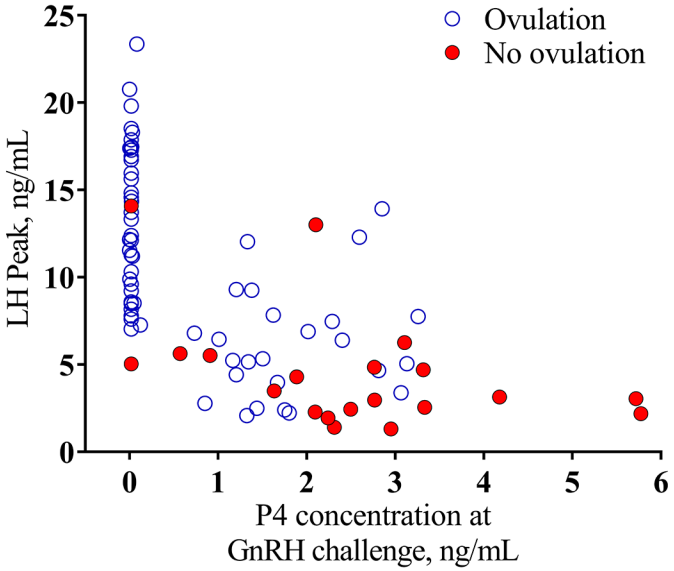

Figure 4. Individual distribution of cows that ovulated (blue open circles) or not (red circles) in response to treatment with 100 μg of gonadorelin (GnRH) on d 0, plotted in relation to their circulating progesterone (P4) concentration at GnRH treatment and amplitude of the GnRH-induced LH peak.

n = 7), the new CL had completely disappeared by d 14, which was considered full structural luteolysis (Table 4).

Circulating P4 concentrations were also measured on d 5, 7, and 14 in cows that ovulated to the GnRH treatment. For these analyses, one cow from group NoCL_IPI and one from group NoCL_NoIPI were considered outliers and were excluded. Similar to what was observed on d 3 (Table 1), on d 5, there was still a clear increase in P4 concentrations due to the presence of the original CL ($P < 0.01$) and the IPI insertion ($P < 0.01$; Table 5). In addition, an interaction was detected on d 5 ($P < 0.01$). Although it was not possible to distinguish the real contribution of each P4 source (original CL, new CL, or IPI) on circulating P4 concentrations within experimental groups, it is possible to infer that, from d 3 to 5, the new CL started to contribute some circulating P4 concentrations (less than 1 ng/mL), as observed in cows from group NoCL_NoIPI. Moreover, comparing the mean circulating P4 of each experimental group, it is possible to infer that the IPI increased P4 concentrations approximately 2 ng/mL on d 5 (Table 5). Thereafter, on d 7, only the main effect of presence of original CL was maintained ($P < 0.01$), whereas a tendency was observed for the IPI insertion effect ($P = 0.08$), and no interaction was detected ($P = 0.95$). On d 7, cows from groups with original CL still had a 3-fold greater circulating P4 concentration compared with cows from groups without the original CL. A

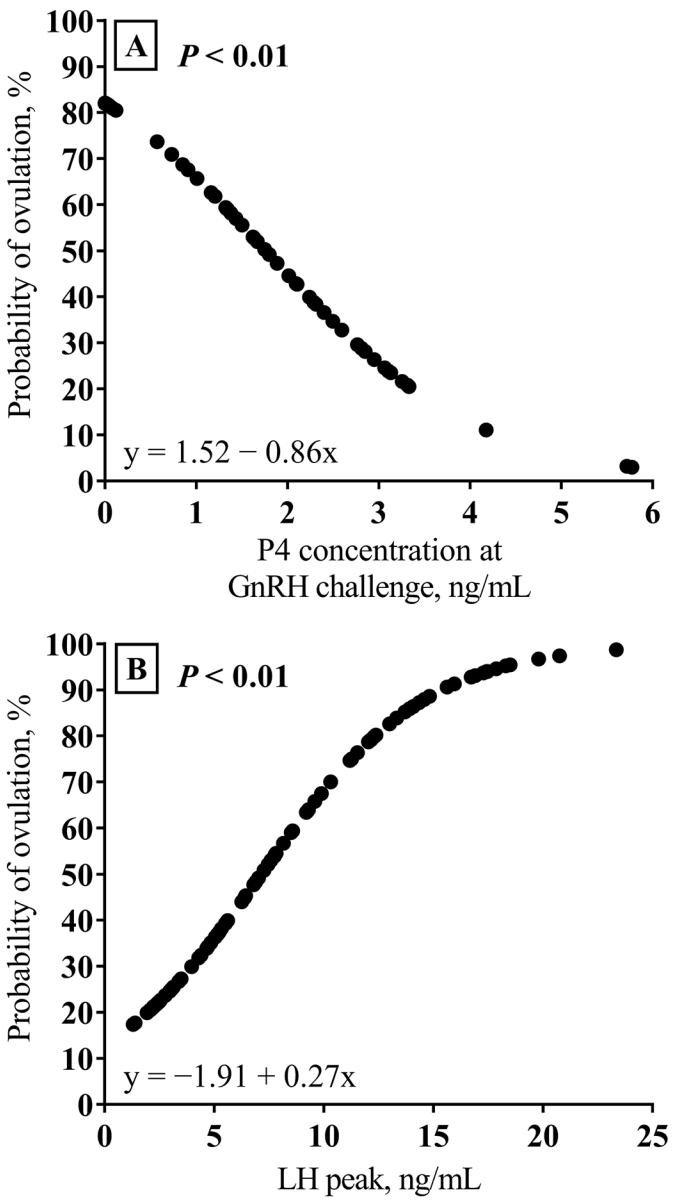

Figure 5. Probability of ovulation after treatment with 100 μg of gonadorelin (GnRH) on d 0 in relation to circulating progesterone (P4) concentrations at the time of GnRH (A); and in relation to the amplitude of the GnRH-induced LH peak (B).

small difference (~0.5 ng/mL; $P = 0.08$) was observed for circulating P4 between cows that received or not the IPI (Table 5). Last, on d 14, consistent with what was observed in the CL volume analyses, an interaction was detected ($P < 0.01$). On this day, the mean circulating P4 decreased in all groups compared with d 7, except in group NoCL_NoIPI, in which circulating P4 had a 2.3-fold increase, reaching 4.4 ng/mL (Table 5).

Because the presence of the IPI contributed to circulating P4 concentrations, it was not possible to de-

Table 4. Dynamics of the original and new corpus luteum (CL) analyzed for each experimental group from d 7 to 14 of the experiment, in cows that ovulated to the GnRH treatment on d 0[1]

	CL		No CL		*P*-value[2]		
Item	IPI (n = 11)	No IPI (n = 14)	IPI (n = 18)	No IPI (n = 21)	CL	IPI	CL × IPI
Original CL							
Volume on d 7, cm^3	4.2 ± 0.4	5.3 ± 0.4	—	—	—	0.10	—
Volume on d 14, cm^3	1.8 ± 0.4	1.6 ± 0.2	—	—	—	0.90	—
Volume change from d 7 to 14, %	–57 ± 9	–68 ± 4	—	—	—	0.23	—
Structural luteolysis d 7 to 14,[3] % (n/n)	63.6 (7/11)	85.7 (12/14)	—	—	—	—	—
New CL							
Volume on d 7, cm^3	3.0 ± 0.4^{a,w}	2.5 ± 0.2^{a,z}	3.9 ± 0.4^{d,w}	4.9 ± 0.3^{c,y}	<0.01	0.53	0.05
Volume on d 14,[4] cm^3	2.3 ± 0.7^{a,w}	1.1 ± 0.3^{a,z}	2.1 ± 0.7^{d,w}	6.5 ± 0.3^{c,y}	<0.01	0.02	<0.01
Luteolysis[3,4]	1.0 ± 0.4	0.5 ± 0.2	0.5 ± 0.2	—	0.12	0.28	—
No luteolysis[3,4]	3.4 ± 1.1	2.2 ± 0.3	5.4 ± 0.3	6.5 ± 0.3	<0.01	0.98	0.11
Volume change from d 7 to 14,[4] %	–17 ± 32^{a,w}	–45 ± 17^{a,z}	–54 ± 15^{d,w}	+43 ± 10^{c,y}	0.03	0.04	0.01
Luteolysis[3,4]	–81 ± 5	–69 ± 5	–89 ± 5	—	0.42	0.81	—
No luteolysis[3,4]	+27 ± 53	+19 ± 28	+16 ± 25	+43 ± 10	0.38	0.30	0.71
Structural luteolysis d 7 to 14,[3] % (n/n)	45.5 (5/11)	64.3 (9/14)	66.7 (12/18)	0.0 (0/21)	—	—	—
Full structural luteolysis on d 14,[5] % (n/n)	0.0 (0/11)	21.4 (3/14)	38.9 (7/18)	0.0 (0/21)	—	—	—

[a,b]Different letters indicate the interaction (CL × IPI) effect sliced. Effect of IPI insertion within groups with CL ($P < 0.05$).

[c,d]Effect of IPI insertion within groups without CL ($P < 0.05$).

[w,x]Effect of presence of CL within groups that received the IPI ($P < 0.05$).

[y,z]Effect of presence of CL within groups that did not receive the IPI ($P < 0.05$).

[1]Values presented as mean ± SEM or as percentage.

[2]Treatment effects: CL = presence of a 7-d-old CL at GnRH treatment; IPI = insertion of an intravaginal progesterone implant at GnRH treatment; CL × IPI = interaction between presence of CL and IPI insertion.

[3]Structural luteolysis defined as volume of the original or new CL decreased >50% from d 7 to 14.

[4]To better distinguish the effect of structural luteolysis on new CL volume, mean volume of CL on d 14 and volume change from d 7 to 14 are also presented separately for cows that underwent luteolysis or not.

[5]Full structural luteolysis defined as new CL had completely disappeared by d 14.

termine the occurrence of functional luteolysis between d 7 and 14 by the same criterion that was used on d 0 (circulating P4 <0.2 ng/mL). Therefore, in this specific analysis, functional luteolysis was defined as a decrease in circulating P4 concentration of more than 50% from d 7 to 14. This criterion was used for all experimental groups. Table 5 shows the incidence of functional luteolysis determined in each group. Consistent with the

Table 5. Effect of presence of corpus luteum (CL), intravaginal progesterone (P4) implant (IPI) insertion, or both on circulating P4 concentrations on specific days of the study, considering only cows that ovulated to GnRH treatment on d 0[1]

	CL		No CL		*P*-value[2]		
Item	IPI (n = 11)	No IPI (n = 14)	IPI (n = 19)	No IPI (n = 21)	CL	IPI	CL × IPI
Circulating P4 on d 5, ng/mL	6.2 ± 0.6^{a,w}	4.2 ± 0.3^{b,y}	2.5 ± 0.1^{c,x}	0.7 ± 0.1^{d,z}	<0.01	<0.01	<0.01
Circulating P4 on d 7, ng/mL	5.9 ± 0.4	5.2 ± 0.5	2.3 ± 0.2	1.9 ± 0.2	<0.01	0.08	0.95
Circulating P4 on d 14,[3] ng/mL	3.0 ± 0.8^{a,w}	0.8 ± 0.5^{b,z}	1.7 ± 0.4^{d,x}	4.4 ± 0.3^{c,y}	<0.01	0.73	<0.01
Luteolysis[3,4]	1.3 ± 0.2	0.1 ± 0.0	0.7 ± 0.1	—	0.01	<0.01	—
No luteolysis[3,4]	6.0 ± 0.7	4.8 ± 0.7	3.4 ± 0.6	4.4 ± 0.3	0.05	0.86	0.16
Functional luteolysis d 7 to 14,[4] % (n/n)	63.6 (7/11)	85.7 (12/14)	68.4 (13/19)	0.0 (0/21)	—	—	—

[a,b]Different letters indicate the interaction (CL × IPI) effect sliced. Effect of IPI insertion within groups with CL ($P < 0.05$).

[c,d]Effect of IPI insertion within groups without CL ($P < 0.05$).

[w,x]Effect of presence of CL within groups that received the IPI ($P < 0.05$).

[y,z]Effect of presence of CL within groups that did not receive the IPI ($P < 0.05$).

[1]Values presented as mean ± SEM or as percentage.

[2]Treatment effects: CL = presence of a 7-d-old CL at GnRH treatment; IPI = insertion of an IPI at GnRH treatment; CL × IPI = interaction between presence of CL and IPI insertion.

[3]To better distinguish the effect of functional luteolysis on circulating P4 concentrations on d 14, mean circulating P4 is also presented separately for cows that underwent luteolysis or not.

[4]Functional luteolysis defined as decrease in circulating P4 ≥50% from d 7 to 14.

decrease in the volume of the new CL, more than 60% of the cows had undergone luteolysis in groups CL_IPI, CL_NoIPI, and NoCL_IPI by d 14, whereas no cow from group NoCL_NoIPI had functional luteolysis. In addition, to better distinguish the effect of functional luteolysis on circulating P4 concentrations measured on d 14, the mean circulating P4 is also presented separately for cows that underwent functional luteolysis or did not undergo luteolysis, within each group (Table 5). Interestingly, even in cows that did not have functional luteolysis detected, the functionality of the new CL was notably reduced by the IPI insertion, as observed by comparing circulating P4 in group NoCL_IPI vs. NoCL_NoIPI. Despite the presence of the IPI, the mean circulating P4 of group NoCL_IPI was 1 ng/mL lower than in group NoCL_NoIPI, confirming that the CL in the NoCL_NoIPI group had greater P4 production.

Figure 6 illustrates the original and new CL dynamics, and circulating P4 concentrations over time in the subset of cows evaluated. Only cows that ovulated to the GnRH challenge on d 0 are represented in this figure (CL_IPI: n = 6; CL_NoIPI: n = 9; NoCL_IPI: n = 8; and NoCL_NoIPI: n = 11). Within each experimental group, cows were retrospectively classified as having undergone functional luteolysis or not, following the same criterion previously presented, and then average values are presented. In groups with the original CL (CL_IPI and CL_NoIPI), luteolysis occurred after d 11 in all cows that underwent luteolysis. In contrast, in group NoCL_IPI, 2 cows underwent luteolysis earlier, on d 7 and 9, and 3 on d 13; whereas no cows underwent luteolysis in group NoCL_NoIPI.

DISCUSSION

The current study evaluated the effect of circulating P4 concentrations produced by a CL or delivered by an IPI inserted at the time of treatment with a conventional dose of gonadorelin (100 μg), on the GnRH-induced LH release, ovulatory response, and subsequent CL development in nonlactating Holstein cows. The experimental design produced P4 concentrations that are normal for d 7 of the estrous cycle in the CL groups or produced low circulating P4, typical of an anovular cow or a cow in proestrus in the NoCL groups (Sartori et al., 2004). Before the GnRH challenge (h 0), although all groups had low circulating LH concentrations (<1 ng/mL), the groups without CL had greater basal LH concentrations compared with groups with CL. This difference can be explained by the dramatic decrease in circulating P4 concentrations from d −1.5 to 0, due to the PGF treatments in groups NoCL_NoIPI and NoCL_IPI, as previously described (Clarke and Pompolo, 2005, Colazo et al., 2008). Treatment with GnRH in synchronized cows with an ~7-d-old DF mimics the conditions that are present when Ovsynch begins after a presynchronization program, such as Double-Ovsynch (Bello et al., 2006; Souza et al., 2008). As expected, the DF growth rate from d −1.5 to 0 was greater in the NoCL groups (PGF-treated groups; NoCL_IPI and NoCL_NoIPI) than in CL groups, consistent with the results by Cerri et al. (2011). Nevertheless, this short period, 36 h, under low circulating P4 before the GnRH challenge was not enough to trigger an endogenous preovulatory GnRH peak, and none of the cows had estrus before the GnRH challenge. Thus, this experimental design allowed valid examination of our 4 experimental hypotheses.

The first hypothesis, that the circulating P4 produced by a 7-d-old CL would suppress the GnRH-induced LH surge, was fully supported. In the absence of a functional CL on d 0, circulating P4 was below 0.2 ng/mL, resulting in a much greater GnRH-induced LH surge as measured at all time points. During greater P4 concentrations provided by a CL, the LH concentration was much lower at all times that were evaluated, and the maximum amplitude observed was substantially suppressed (2.5-fold greater in low than high P4) compared with groups without CL. Several studies have reported the suppressive effect of high circulating P4 concentrations on the GnRH-induced LH peak (Hapgood et al., 2005; Colazo et al., 2008; Giordano et al., 2012a; Lima et al., 2013; Motta et al., 2020). Previous studies have reported that steroid hormones can regulate the sensitivity of the pituitary gland to GnRH, perhaps due to P4 suppressing expression of GnRH receptors in the pituitary gland (Rispoli and Nett, 2005; Nett et al., 2002; Stevenson and Pulley, 2016). Although, we did not measure E2 in this study, it is likely that high circulating P4 concentrations would also suppress circulating E2 from the DF, even though the follicle was at the same stage of development (d 7). Our previous research in Holstein cows (Motta et al., 2020) and previous research in cows in anestrus (Looper et al., 2003) support the idea that circulating E2 (stimulatory) and P4 (inhibitory) are critical for regulating the expression of GnRH receptors and for pituitary LH secretion in response to GnRH. Because the response of gonadotrophs to GnRH is directly correlated with the number of GnRHR on the cell surface during the ovine estrous cycle (Wise et al., 1984), it is logical that at least part of the P4-induced decrease in GnRH responsiveness is due to decreased GnRHR numbers. Indeed, a decrease in GnRHR mRNA and proteins has been observed following P4 treatment of cultured ovine pituitary cells (Laws et al., 1990; Wu et al., 1994) and in vivo (Turzillo et al., 1995; Nett et al., 2002). These effects of P4 on GnRHR could be through direct inhibitory effects of P4 on the

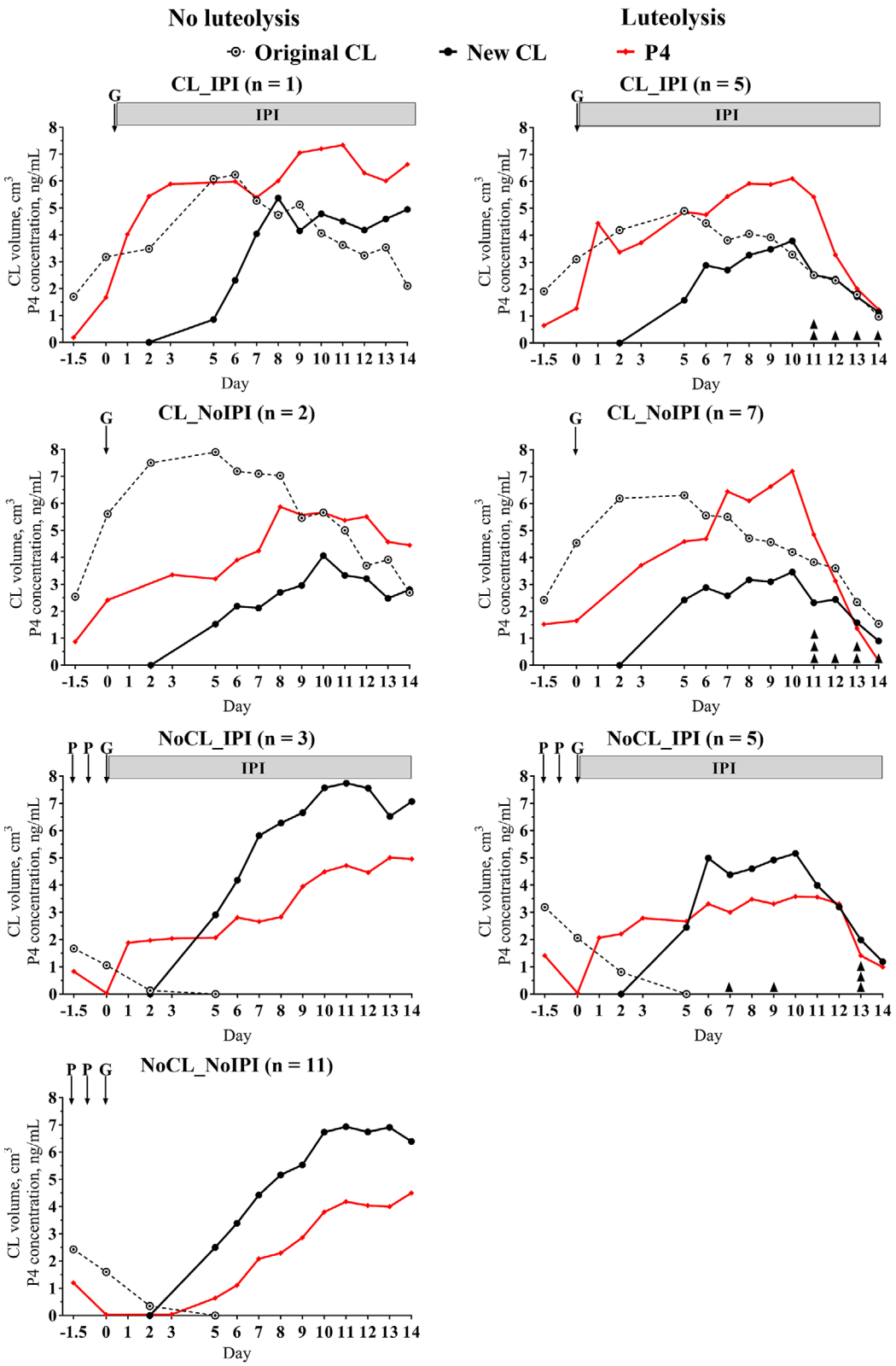

Figure 6. Original and new corpus luteum (CL) volume and circulating progesterone (P4) concentrations from d −1.5 to 14 of the experimental design, in cows that ovulated to GnRH treatment on d 0, classified as undergoing functional luteolysis (right side) or not undergoing luteolysis (left side), within each experimental group. Timing of $PGF_{2\alpha}$ (P), GnRH (G), and intravaginal P4 implant (IPI) treatments are shown. Time of functional luteolysis (>50% decrease in P4) for individual cows is represented by a dark triangle (▲) above the timeline in each group.

pituitary gonadotrophs because they can be observed in cultured pituitary cells or could be through suppression of the stimulatory effects of E2 on GnRHR. Alternatively, P4 may be acting indirectly through suppression of hypothalamic GnRH pulse frequency (Clarke and Pompolo, 2005), and GnRH pulses can stimulate GnRHR expression, even in the presence of high P4 (Turzillo et al., 1995; Rispoli and Nett, 2005; Kanasaki et al., 2013). In addition, pathways downstream of the GnRHR, including second messenger pathways and pituitary LH content, may also be key aspects of the P4-induced decrease in GnRH-stimulated LH secretion (Rispoli and Nett, 2005). Thus, consistent with our first hypothesis and previous research, the greater the P4 concentrations at the time of GnRH treatment the less LH is released in the LH surge.

The second hypothesis was that the insertion of an IPI at the time of GnRH treatment would cause a rapid increase in circulating P4 concentrations, sufficient to negatively affect the GnRH-induced LH surge. This hypothesis was not supported by our results. Although following IPI insertion there was rapid increase in circulating P4 concentrations by 1 h, as previously described (Cerri et al., 2009a), there was no effect on GnRH-induced LH release either in the cows with CL or in those without CL. The circulating LH concentrations over time and the maximum LH amplitude were very similar between cows receiving or not the IPI. These results are consistent with the idea that the effects of P4 must be present before the GnRH treatment and that there is no acute response to P4 after GnRH treatment. Future experiments could be designed to evaluate how long a period of P4 exposure is required to induce the inhibitory mechanisms on GnRH-induced pathways. Thus, simultaneous treatment with P4 at the time of GnRH treatment does not appear to reduce the effectiveness of GnRH, in terms of the magnitude of the LH surge.

Furthermore, regardless of CL presence or IPI insertion, results from our study support our third hypothesis, that the peak of the LH surge was related to whether the GnRH treatment induced ovulation. Analysis of individually distributed LH peak according to circulating P4 at the time of GnRH challenge clearly demonstrated the suppressed LH release under high P4 concentrations, despite variability among cows. For instance, all cows with circulating P4 $\leq$ 0.5 ng/mL had a GnRH-induced LH peak >5 ng/mL, of which 70% (29/41) were above 10 ng/mL, whereas only 10% (4/39) of cows with circulating P4 $\geq$ 1 ng/mL had an LH peak >10 ng/mL. Moreover, almost all cows that did not ovulate had an LH peak <5 ng/mL. These results are consistent with what was previously reported (Giordano et al., 2012a). Additionally, the logistic models created to predict the probability of ovulation after a treatment with 100 μg of gonadorelin, as a function of the circulating P4 at GnRH treatment and of the LH peak amplitude, corroborate previous studies that reported ovulatory response to this treatment in lactating cows and heifers (Colazo et al., 2008; Lima et al., 2011; Giordano et al., 2012b). Moreover, in our study, the ovulatory response was not affected by the IPI insertion at the time of GnRH, unlike what has been suggested in previous studies (Galvão et al., 2004; Stevenson et al., 2008). Interestingly, Lima et al. (2013) evaluated the GnRH-induced LH release and ovulatory response of Holstein heifers with or without CL, receiving an IPI 24 h before the treatment with 100 μg of gonadorelin. In the group Low P4 (without CL), luteolysis was induced by 2 $PGF_{2\alpha}$ administrations, at the time of IPI insertion and 12 h later. Despite the high LH peak reported in this group (31.3 ng/mL), the ovulatory response was low (~48%), but greater than in heifers with CL (~19%). These findings suggest that, even in absence of a functional CL at the time of GnRH treatment, the circulating P4 provided by the IPI inserted 24 h before GnRH could impair ovulation. However, our results indicate that insertion at the time of GnRH treatment does not impair ovulatory response. Perhaps the discrepancy lies in the function of the DF at the time of GnRH treatment in the 2 studies.

The present study also evaluated the development of the new CL, formed after the GnRH-induced ovulation. The findings supported our fourth hypothesis, confirming that elevated P4 concentrations, either from an original CL or from an IPI, compromise the development and functionality of the new CL. There are 2 aspects to this impaired development of the new CL: reduced CL size/function observed on d 7 possibly mediated by reduced LH pulses, and earlier regression of the CL as observed on d 14 and in the cows monitored by daily ultrasound/P4 during the luteal phase, possibly due to uterine $PGF_{2\alpha}$ secretion. As expected, cows that ovulated in the presence of the original CL developed a smaller new CL compared with cows without the original CL at the time of GnRH treatment, despite having ovulated a DF of similar diameter at time of GnRH treatment. Moreover, although it was not statistically compared, it is possible to observe that the mean volume of the new CL on d 7, considering cows from groups CL_NoIPI and CL_IPI that ovulated to GnRH, was approximately 35% smaller (2.7 vs. 4.2 cm^3) than the original CL on d 0 (both 7 d old at time of measurement). In our experimental model, the GnRH treatment on d 0 in cows from groups CL_NoIPI and CL_IPI acted as an inducer of accessory CL, on d 7 of the estrous cycle (Souza et al., 2009a; Vasconcelos et al., 2011). Hence, the ovulation that generated the new CL in these groups was not preceded by a proestrus period

(Wiltbank et al., 2014), and the development of this CL occurred under high circulating P4 concentrations since its beginning. Moreover, even in group NoCL_IPI, in which cows underwent luteolysis and had a period with very low circulating P4 before the GnRH-induced ovulation, the insertion of the IPI negatively affected the development of the new CL. Niswender et al. (2000) indicated that CL development in ewes is mediated by LH and growth hormone, and LH induces expression of vascular endothelial growth factors, which probably is associated with the proliferation of luteal endothelial cells. Therefore, results from our study support the idea that elevated P4 concentration (promoted by the original CL or by the IPI) during the development of the early new CL can impair its growth, resulting in a smaller CL. Similar results were previously reported from studies in beef (Parr et al., 2017) and dairy heifers (Burke et al., 1994).

Studies also reported that the suppression of LH pulse frequency during development of the CL may negatively affect the functionality of the CL (Peters et al., 1994; Skarzynski et al., 2013). In this sense, in our study, it is possible that the elevated P4 concentrations promoted by the original CL or by the IPI had reduced LH pulse frequency during the early development of the new CL, impairing also its functionality, similar to what was reported in ewes (Letelier et al., 2011; Christensen et al., 2012). Unfortunately, the model used in the present study did not allow differentiation of the source of the circulating P4 by each CL (original or new) or the IPI. Nonetheless, by analyzing the circulating P4 concentrations observed at distinct moments after ovulation, it is possible to infer the functionality of the new CL according to each experimental group. On d 3 and 5, the mean circulating P4 among groups are consistent with what would be expected from the CL (Sartori et al., 2004) and from this specific IPI in Holstein cows (Silva et al., 2021). However, on d 7, the IPI is expected to contribute with ~1.5 ng/mL to the circulating P4 concentrations (Silva et al., 2021), but the circulating P4 decreased from d 5 to 7 in the 2 IPI groups while it increased about 1 ng/mL in the groups without IPI, making it likely that the presence of the IPI reduced P4 production by the CL. Thus, both the CL volume and circulating P4 on d 7 are consistent with a negative effect of the elevated P4 on CL size/function, probably by reducing LH pulse frequency during its early development.

Related to the second aspect of impaired luteal function, timing of luteolysis, in all groups except group NoCL_NoIPI, in at least half of the cows, the new CL regressed between d 7 and 14. Based on the criteria used in this study, these cows were classified as undergoing structural and functional luteolysis. Based on the subset of cows evaluated daily, it is possible to observe that, in groups CL_NoIPI and CL_IPI, the functional luteolysis occurred after d 11, accompanied by a substantial decrease in volume of the new CL and circulating P4. This would be the expected time for luteolysis of the original CL because experimental d 11 corresponds to estrous cycle d 18 (GnRH on d −7) and this would coincide with the normal time of luteolysis in Holstein cows (Sartori et al., 2004; Wiltbank et al., 2018; Mezera et al., 2019). Thus, both the original and new CL regress in response to the $PGF_{2\alpha}$ generated from the uterus at the expected time. Nevertheless, and particularly important for understanding our fourth hypothesis, 68% (13/19) of cows from group NoCL_IPI underwent luteolysis between d 7 and 14, a time that is much earlier than expected luteolysis and these cows did not have an original CL. Based on the daily evaluation, the time of luteolysis in these cows varied from d 7 to 13. In this sense, 2 feasible explanations were considered for the early regression of new CL in group NoCL_IPI. The first is that the short cycle had been induced by the supplementation of P4 at the early stages of CL development, consistent with what has been reported (Burke et al., 1994; Parr et al., 2017). Alternatively, it is possible that the lack of an adequate proestrus after the induced ovulation induced a short cycle, as described previously (Peters and Pursley, 2003; Rantala et al., 2009). The first explanation seems to be more reasonable because cows from the group NoCL_NoIPI, submitted to the same proestrous period, did not have short cycle or early luteolysis. Thus, early luteolysis in the group NoCL_IPI is likely due to the early rise in P4 from the IPI resulting in earlier downregulation of P4 receptors in the uterine endometrium and early secretion of $PGF_{2\alpha}$ from the uterus as described for other models of short cycles (Copelin et al., 1989; Hunter, 1991; Garverick et al., 1992; Wiltbank et al., 2016).

CONCLUSIONS

The circulating P4 concentrations produced by a 7-d-old CL at the time of GnRH treatment negatively affected the GnRH-induced LH release, ovulatory response, and subsequent CL development in nonlactating Holstein cows. The insertion of an IPI at the same time as GnRH treatment did not affect the GnRH-induced LH release nor ovulatory response, but compromised the subsequent CL development and functionality, inducing short cycles and regression of the new CL in cows without CL at the time of GnRH treatment.

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