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Luteal activity following follicular drainage of subordinate follicles for twin pregnancy prevention in bi-ovular dairy cows

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ABSTRACT

Twin pregnancy is undesirable in dairy cattle. This study examines luteal activity following ultrasound-guided puncture and drainage of the smaller pre-ovulatory follicle at timed AI in cows with a pre-ovulatory follicle in each ovary. Luteal activity was determined through Doppler ultrasonography and plasma progesterone (P4) concentrations. The effects of GnRH treatment on Day 7 post-AI on subsequent luteal activity were also assessed. Two study groups were established: a control group of 29 cows and a follicular drainage (FD) group of 28 cows. After drainage, all cows developed a corpus luteum (CL) in the drained ovary. On Day 21 post-AI, drainage-induced CL and fellow CL were similar in terms of size and vascularization. According to a GLM repeated measures analysis of variance ($P < 0.001$), non-treated drained cows had lower P4 concentrations on Day 21 post-AI than non-treated non-drained cows, whereas GnRH treated cows, both drained and non-drained, showed the highest P4 concentrations at this time point. Twin pregnancy was recorded in 3 of the 8 pregnant control cows, whereas no twins were observed in the FD group. Our results indicate that luteal structures following follicular drainage were functional. As for the presence of an additional CL, this could suggest a reduced risk of pregnancy loss. In addition, luteal activity was significantly increased following GnRH treatment on Day 7 post-AI in drained cows.

1. Introduction

Although infrequent in monotocous mammalian species, natural multiple births involve in most cases the simultaneous formation of two or more ovulatory follicles on either the same ovary or on both ovaries. The birth of twins was probably a basis for the early development of concepts of human fertility (López-Gatius and Hunter, 2018a) and is very welcome for the economy of beef cattle breeding (Echternkamp et al., 2004; Hashiyada, 2017). However, twin pregnancy is not desirable in dairy cattle and its economic burden in the range of $97 to $225 is dependent on the type of twin pregnancy (unilateral vs. bilateral), parity, and the days in milk when the twin pregnancy occurs (Mur-Novales et al., 2017). In fact, twin pregnancy is the main non-infectious factor that compromises pregnancy maintenance during the first trimester of gestation and is logically related to subsequent twinning (López-Gatius and García-Ispierto, 2010; López-Gatius et al., 2017). While spontaneous embryo reduction rates of 11.2% to 28.4% have been reported occurring at around 28–40 days of gestation (López-Gatius and Hunter, 2005; Silva-del-Río et al., 2009; López-Gatius et al., 2010), twin pregnancy losses can exceed 50%, especially during the warm period of the year in some countries (López-Gatius et al., 2004; Andreu-Vázquez et al., 2011). Apart from pregnancy loss, the reproductive performance and productive lifespan of a cow delivering twins are greatly reduced (Beerepoot et al., 1992; Bicalho et al., 2007; Andreu-Vázquez et al., 2012b). Twin pregnancy rates can exceed 18% in some herds (Andreu-Vázquez et al., 2012a), and two strategies have been tested to mitigate their negative effects: hormone therapy or artificial embryo reduction (López-Gatius et al., 2017; López-Gatius and Hunter, 2017b). In effect, GnRH treatment at the time of pregnancy diagnosis increases pregnancy survival and is accompanied by an

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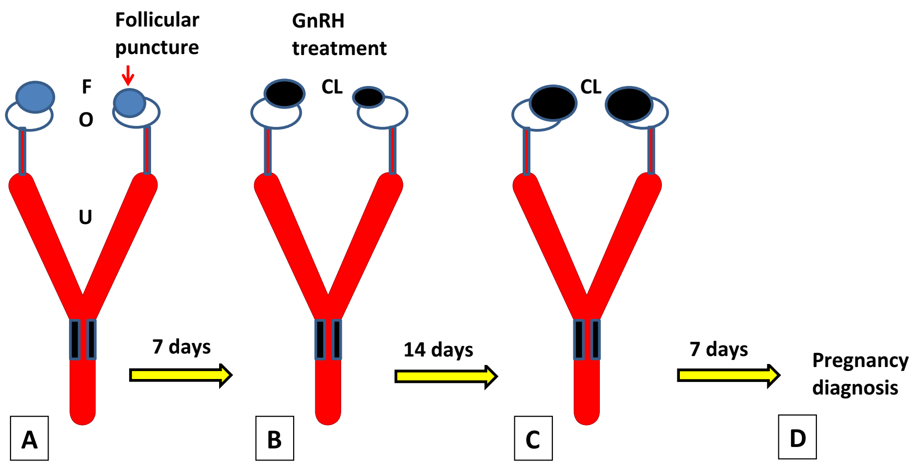


Fig. 1. Experimental design on cows with a pre-ovulatory follicle in each ovary. Puncture and drainage of the smaller pre-ovulatory follicle was performed at timed AI in the drainage group (A). Ovulation and luteal activity were assessed on Day 7 post-AI. Luteal activity was determined through corpora lutea measurements and plasma progesterone determinations. A GnRH dose was administered at this time point in the treatment group of drained and non-drained cows (B). Luteal activity was determined through Doppler ultrasonography, corpora lutea measurements and plasma progesterone determinations on Day 21 post-AI (C). Pregnancy diagnosis was performed on Day 28 post-AI (D). F: follicles; CL: corpora lutea; O: ovaries; U: uterus.

increase in the twin reduction rate (Garcia-Ispierto and López-Gatius, 2018).

Other proposed approaches to reduce twinning in cows on Days 28–41 of gestation consist of manual rupture of the amniotic vesicle and transvaginal ultrasound-guided aspiration of allanto-amniotic fluid or intra-luteal instillation of $PGF_{2\alpha}$ in one of the two corpora lutea (López-Gatius, 2005; Andreu-Vázquez et al., 2012c; López-Gatius and Hunter, 2016). However, although inducing twin reduction avoids the negative effects of twinning, these interventions may increase the risk of pregnancy loss.

To eliminate the risk of twin pregnancy without reducing fertility, the puncture and drainage of subordinate follicles at the time of insemination has recently proved to be an efficient procedure (López-Gatius and Hunter, 2018b). This technique overcomes the risk of pregnancy loss related to the process of induced twin reduction and increases the incidence of additional corpora lutea. In this latter study, all drained follicles developed as a corpus luteum 7 days later (López-Gatius and Hunter, 2018b). Draining with no suction supposedly spares a sufficient number of granulosa cells in the follicle which could subsequently give rise to luteal tissue. If these additional luteal structures are functional they may reduce the subsequent risk of pregnancy loss. In effect, a number of corpora lutea exceeding the number of embryos has emerged as a strong factor promoting maintenance of a pregnancy (López-Gatius, 2012).

The objective of the present study was to assess luteal activity based on Doppler ultrasonography (Acosta et al., 2003; Matsui and Miyamoto, 2009) and plasma progesterone (P4) concentrations following the puncture and drainage of subordinate follicles at timed AI in bi-ovular cows. A second goal was to evaluate the effect of GnRH treatment on Day 7 post-AI on subsequent luteal activity and fertility.

2. Materials and methods

2.1. Experimental animals

All procedures were approved by the Ethics Committee on Animal Experimentation of the University of Lleida (license number CEEA.06-01/12). The study population was a commercial dairy herd of Holstein-Friesian lactating dairy cows in northeastern Spain. During the study period (November 2017 to April 2018), the mean number of lactating cows in the herd was 240, and mean annual milk production was 10,190 kg per cow. In our geographical area, a clear negative influence of heat stress from May to September on the reproductive performance of lactating dairy cows has been extensively described (López-Gatius, 2003; García-Ispierto et al., 2007). In effect, ovulation failure increases dramatically under heat stress conditions (López-Gatius et al., 2005; López-Gatius and Hunter, 2017a). Thus, to reduce the number of pre-ovulatory follicles failing to ovulate, this study was performed during the cool period of the year (November to April). Cows were fed complete rations and milked twice daily. Only healthy cows free of detectable reproductive disorders and free of clinical diseases during the study period (days −5 to +28 from insemination) were included. Exclusion criteria were the following: mastitis, lameness, digestive disorders and pathological abnormalities of the reproductive tract detectable by ultrasonography. Cows were selected from groups synchronized for fixed-time insemination (Garcia-Ispierto and López-Gatius, 2014). Cows were treated with a controlled internal drug release insert (CIDR containing 1.38 g of P4; Zoetis, New York, NY, USA) plus a GnRH agonist (dephereline: 100 μg gonadorelin acetate [6-D-Phe] i.m; Gonavet Veyx, Ecuphar, Barcelona, Spain) upon CIDR insertion. The CIDR was left in place for 5 d, and these animals were also given cloprostenol (500 μg i.m.; PGF Veyx Forte, Ecuphar, Barcelona, Spain) on CIDR removal. Twenty-four h and 36 h later, the cows received a second cloprostenol dose and a second GnRH dose, respectively, and were inseminated 50–56 h after CIDR removal. The GnRH agonist used in the present study was selected for its high efficiency in improving luteal function (García-Ispierto et al., 2019). Cows with a 2.5–3.5 body condition score on a scale of 1 to 5 (Edmondson et al., 1989) and producing > 30 kg milk per day were selected for follicular drainage at the time of insemination. A combination of ultrasonography and manual rectal palpation was used to confirm a cow was in estrus and ready for service (López-Gatius and Camón-Urgel, 1988; López-Gatius and Hunter, 2017a). Only cows with at least two follicular structures equal to or larger than 12 mm in diameter in the absence of a corpus luteum, and with the two largest follicles located one on each ovary, were included in the study. Since double ovulation has been related to the least possible size differences between the larger and smaller follicle irrespective of the individual diameter of each follicle López-Gatius et al., 2018), only cows with a size difference under 2 mm between the two co-dominant follicles were included in the study.

2.2. Follicular drainage and experimental design

Cows were assigned in chronological order of estrus synchronization to a control ($n = 30$) or follicular drainage (FD) ($n = 30$) group (Fig. 1). Follicular puncture and drainage of the subordinate follicle in the FD group was performed by ovum pick-up procedures as previously described (López-Gatius and Hunter, 2018b). Briefly, a portable B-mode ultrasound scanner (E.I. Medical IBEX LITE; E.I. Medical Imaging, Loveland CO, USA) equipped with a convex 5–10 MHz (E.I. Medical IBEX MC8.0 10-6 Microconvex; E.I. Medical Imaging, Loveland CO, USA) transvaginal transducer was used for draining through a sterile 19G 25-mm long needle located in the tip of the transducer's metal guide. The vulva and the perineal region of the cow and the metal guide were washed in an iodine solution and the transducer probe was coated with a sterile preservative. Then, the metal guide containing the needle was introduced into the dorsal vaginal fornix, which was to the left or right of the cervix depending on the side of the subordinate follicles. Next, the ovary was positioned transrectally against the tip of the transducer probe so that the follicle was separated only by the vaginal wall. The vaginal wall was then pierced in a cranial direction through the fornix with the needle and introduced into the follicular antrum (López-Gatius and Hunter, 2018b). Follicular contents flowed rapidly through the metal guide with no suction. In the FD group, the largest follicle was considered the dominant follicle and was not drained. Cows showed no signs of discomfort during intra-follicular puncture.

Cows were inseminated immediately after follicular drainage with thawed semen from a single ejaculate. In 14 drained and 14 non-drained cows, dephereline treatment was randomly given on Day 7 post-AI. Ovulation, determined in the FD group by the presence of a corpus luteum in the dominant follicle, and in the control group by the presence of one or two corpora lutea, was assessed 7 days post-AI. Corpus luteum size was recorded in ovulating ovaries and the luteal structure as a corpus luteum in drained ovaries 7 days post-AI taken as the mean of two measurements approximating the greatest length and width. In the case of cavity CL, the mean value of the luteal wall was also recorded. Pregnancy diagnosis was performed by ultrasound at 28 days post-AI (Fig. 1). All gynecological examinations and inseminations were performed by the same operator.

Luteal activity was evaluated through plasma P4 concentrations determined on Days 7 and 21 post-AI and through Doppler-ultrasonography on Day 21 post-AI.

2.3. Luteal activity measurements

Blood samples were collected on Days 7 and 21 post-AI from the coccygeal vein into two heparinized vacuum tubes (BD VacutainerTM; Becton-Dickenson and Company, Plymouth, UK), centrifuged within 20 min (10 min, 1600 g) and the plasma stored at −20 °C until analysis.

A commercial enzyme-linked immunosorbent assay (ELISA) kit was used to determine plasma progesterone concentrations (Ridgeway Scientific, Alvington, Gloucestershire, UK). The sensitivity of the assay was 0.15 ng / ml. Samples were tested in duplicate, and all samples were analyzed in a single assay (intra-assay coefficient of variation, 6%).

Both ovaries were examined by color Doppler ultrasonography on Day 21 post-AI (Zonare Medical Systems Inc., USA equipped with a 7.5 MHz transducer) and surfaces of corpora lutea scanned and digitalized in a video. Images of cross-sections of each CL were also recorded. The diameter (mean of two measurements) and area were obtained in the ultrasound image that visually represented the largest CL. In the case of CLs with a cavity, the latter was measured and subtracted to obtain the area of luteal tissue. Three cross-sectional images covering the largest areas of the respective CL were finally recorded, and the most vascularized picture selected for each corpus luteum. All corpora lutea were digitalized in B-mode, power and color Doppler mode. All images were digitized in DICOM format. Fixed pre-installed Doppler system controls were used to exclude variations in recordings.

The color Doppler images were used to analyze vascular areas in the luteal structures. The vascular area was calculated from each selected CL picture using Image J® software (National Institutes of Health, Bethesda, MD, USA). The percentage of vascularized CL (color Doppler area/total CL area × 100) was then determined for each evaluation. All ultrasound analyses were performed by the same operator.

2.4. Data collection and statistical analysis

The following data were recorded in each animal: parturition and treatment dates; parity (primiparous versus multiparous); milk production at AI (low producers < 40 kg versus high producers ≥ 40 kg); days in milk at AI (DIM; < 90 days postpartum versus ≥ 90 days postpartum); follicular size at AI (diameter of the follicles ≥ 12 mm); follicular drainage (control vs FD); treatment on Day 7 post-AI (non-treated vs GnRH group); CL size and number of CL at 7 and 21 days post-AI; CL vascularization on Day 21 post-AI (four classes: no vascularization; < 25%; between 25 and 50%; > 50%); ovary in which follicular or luteal structures were recorded (right versus left ovary); plasma progesterone concentration on Days 7 and 21 post-AI; and conception rate after FTAI. Conception rate was defined as the percentage of cows that became pregnant at FTAI out of the total number of cows in the corresponding group.

The effects of the above-mentioned variables and those of plausible interactions on plasma progesterone concentrations on Days 7 and 21 post-AI were assessed by general linear model (GLM) repeated measures analysis of variance using the SPSS computer package, version 11.5 (SPSS Inc., Chicago, IL, USA).

Differences in CL size on Day 21 post-AI in the control and FD groups and GnRH-treated and non-treated cows were analyzed by the Student's test. Possible significant effects of drainage, treatment and drainage-treatment interaction on the conception rate were explored by Tukey-Kramer multiple comparison tests. Significance was set at $P < 0.05$. Values are expressed as the mean ± the standard deviation (SD).

3. Results

Three cows with three corpora lutea at 7 days post-AI were withdrawn from the study. The final study population was comprised of 57 cows: 29 in the control group and 28 in the FD group. Ovulation failure (absence of a CL 7 days after AI) was registered in 2 control cows. The data collected in these two animals were eliminated from the P4 analyses but maintained for the conception rate analysis. Table 1 shows luteal dynamics on Days 7 and 21 post-AI following drainage and GnRH treatment. Double ovulation was recorded in 12 cows (41.4%) in the control group. After follicular drainage, all cows showed a CL in the drained ovary, whereas the corresponding dominant follicle failed to ovulate in 6 (21.4%) of the 28 FD cows. No pregnancies were produced in these latter 6 cows. On Day 21 post-AI, luteal structures were not detected in 12 cows: 7 in the control group and 5 in the FD group. No pregnancies were recorded in these 12 cows and the P4 data obtained in these animals were removed from the analyses. Of the 28 cows receiving GnRH on Day 7 post-AI, 6 had a GnRH-induced CL on Day 21

Table 1
Luteal patterns and conception rate after both follicular drainage and GnRH treatment (n = 57).

Drainage D0	GnRH D7	2 CL D7 n (%)	0 CL D21 n (%)	Conception rate n (%)	Twins* n (%)
No	No	5/15 (33.3)	4/15 (26.7)	4/15 (26.7)[a,b]	1/4 (25)
	Yes	7/14 (50)	3/14 (21.4)	4/14 (28.6)[a,b]	2/4 (50)
Yes	No	10/14 (71.4)	3/14 (21.4)	3/14 (21.4)[a]	0/3 (0)
	Yes	12/14 (85.7)	2/14 (14.3)	8/14 (57.1)[b]	0/8 (0)

Values with different superscripts within columns denote significant differences detected by the Tukey-Kramer test ($P < 0.05$).
* Values on pregnant cows.

post-AI: 3 in the control and 3 in the FD group (Table 1). The GnRH-induced CL was the third CL in the FD group.

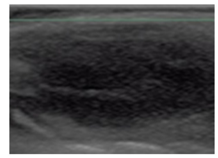
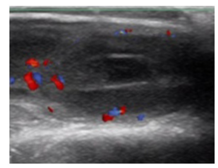
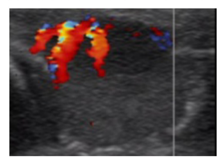
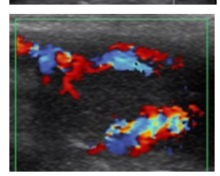
Mean milk production at the time of treatment, DIM at AI and number of lactations, were 44.5 ± 9.5 kg, 123.0 ± 70.1 days, 2.7 ± 1.6 lactations, respectively (mean ± SD). In the GLM repeated measures analysis of variance, parity, milk production and days in milk at AI were found to have no effects on luteal patterns.

Luteal activity was assessed only in cows with at least one CL on Day 7 (n = 55) or Day 21 (n = 45) post-AI. The mean size of the CL on Day 7 post-AI resulting from ovulation (16.2 ± 4 mm, ranging from 8 to 24 mm) was larger ($P < 0.03$, Student's test) than that resulting from drained follicles (8.2 ± 3.5 mm, ranging from 5 to 15 mm). In 22 FD cows that had both ovulating and drainage CL on Day 21 post-AI, no significant differences in size and vascularization between both types of CL could be detected. Similar CL vascularization patterns were also observed in the remaining cows with two CL. The mean size of GnRH-induced CL on Day 21 post-AI (11.1 ± 2.5 mm, ranging from 6 to 20 mm) was significantly smaller ($P < 0.0001$, Student's test) than that of the remaining CL (18.7 ± 6.2 mm, ranging from 8 to 36 mm). Vascularization of the GnRH-induced CL was similar to that of CL partners.

Mean plasma P4 concentrations increased significantly ($P < 0.0001$, Student's test) from Day 7 to 21 post-AI (2.3 ± 1.6 and 3.4 ± 3.1 ng/mL, respectively). General linear model repeated measures of variance revealed that the treatment x drainage interaction had a significant effect (between subject effect repeated measures ANOVA; $P < 0.001$) on plasma P4 concentrations on Day 21 post-AI. Non-treated drained cows had lower P4 concentrations on Day 21 post-AI than those their non-treated non-drained partners, whereas GnRH-treated cows, both drained and non-drained, showed the highest P4 concentrations on Day 21 post-AI (Fig. 2).

Over the course of the study, 19 cows (33.3%) of the 57 cows enrolled became pregnant: 8 control and 11 FD cows. The conception rate was significantly higher ($P < 0.05$) in drained cows treated with GnRH on Day 7 post-AI than non-treated drained cows. Twin pregnancy was recorded in 3 of the 8 pregnant control cows, whereas twins were not registered in the FD group (Table 1). Corpus luteum vascularization on Day 21 post-AI was positively correlated with plasma P4 concentrations and pregnancy rate (Table 2).

Table 2
Sonograms taken on Day 21 post-AI showing different levels of CL vascularization and their corresponding plasma P4 concentrations and conception rates (n = 45).

Vascularization	%	Mean P4 values ± SD (ng/ml)	Conception rate n (%)	GnRH treated n (%)
	0	0.22 ± 0.21 [a]	0/8 (0)[a]	4 (50)
	< 25	1.49 ± 1.90 [a]	0/9 (0)[a]	4 (44)
	25–50	4.92 ± 1.20 [b]	5/13 (38.5)[a,b]	7 (54)
	> 50	6.30 ± 3.05 [b]	14/15 (93.3)[b]	9 (60)

Values with different superscripts within columns denote significant differences detected by the Tukey-Kramer test ($P < 0.001$).

4. Discussion

In the present study, we examined bi-ovular lactating dairy cows with a pre-ovulatory follicle in each ovary. Findings confirm our previous data indicating that puncture and drainage of the smaller pre-ovulatory follicle (subordinate) in these cows at timed artificial insemination serves to avoid a risk of twin pregnancy (López-Gatius and Hunter, 2018b). Further, while showing individual variation in size, induced luteal structures present in the drained ovary in all cows subjected to this technique were found to be functional. On Day 21 post-AI, these drainage-induced CL were similar in size and vascularization to their counterparts of ovulatory origin. Moreover, luteal activity was greatly increased following GnRH treatment on Day 7 post-AI.

In this setting, however, the high risk of ovulation failure of the dominant follicle suggests that the drainage procedure could impair fertility. Accordingly, to improve the chances of selecting double ovulating animals, only cows with a size difference smaller than two mm between the two co-dominant follicles were included in this study (López-Gatius et al., 2018). In addition, the experiment was performed during the cool period of the year, when the risk of ovulation failure clearly decreases (López-Gatius et al., 2005). Six of the 28 (21.4%) drained cows failed to ovulate. We nevertheless anticipate that treatment with GnRH or hCG at the time of insemination will improve the chances of ovulation for the dominant follicle. Further comprehensive studies are needed to confirm this assertion.

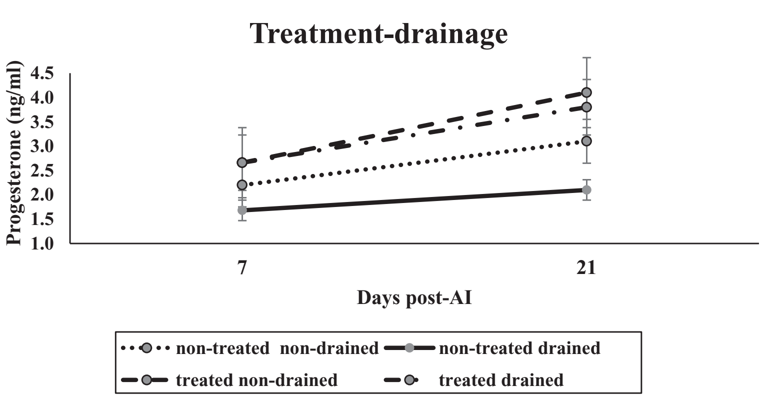


Fig. 2. Mean progesterone concentrations (ng/ml) recorded on Days 7 and 21 post-AI after follicular drainage and GnRH treatment (n = 45). Values differed significantly on Day 21 post-AI between non-treated drained and non-treated non-drained cows and between treated and non-treated cows (between subject effect repeated measures ANOVA; $P < 0.001$).

A further critical point was the impaired functional condition of the luteal structures observed on Day 21 post-AI after follicular puncture. Thus, our results suggest some form of limitation with time after ovulation, seemingly associated with a reduced cell proliferation and steroid secretion potential. Lowest mean plasma P4 concentrations on Day 21 post-AI were recorded in the non-treated drained cows. However, in these animals, treatment with GnRH on Day 7 post-AI significantly increased plasma P4 concentrations and the pregnancy rate, thus overcoming the possible detrimental effect of follicular draining. In effect, we would expect a reduced risk of early fetal loss due to this significant increase in plasma P4 concentrations in response to GnRH treatment (López-Gatius, 2012). Sub-optimal levels of circulating P4 have been extensively related to such losses (Ayad et al., 2007; Karen et al., 2014; Gábor et al., 2016). However, although the conception rate was significantly higher in drained cows treated with GnRH on Day 7 post-AI than in non-treated drained cows, this finding on fertility need to be interpreted with caution. A higher sample size in each group should be required to safely draw any conclusion in future studies.

To assess the luteal function of drainage-induced CL, we used Doppler ultrasonography. This technique proved valuable especially when combined with plasma P4 concentrations, which are useful as an early predictor of pregnancy. Although early pregnancy diagnosis was not an objective of the present study, our results reinforce previous reports on early pregnancy prediction based on luteal activity on Days 14 to 21 post-AI in which CL luteum blood flow area was closely associated with plasma P4 concentrations and subsequent pregnancy, as extensively reported (Herzog et al., 2011; Siqueira et al., 2013; Kanazawa et al., 2017).

As an overall conclusion, puncture and drainage of subordinate follicles at the time of insemination eliminates the risk of twin pregnancy and increases the presence of additional functional corpora lutea. GnRH treatment on Day 7 post-AI greatly improves subsequent luteal function in cows undergoing follicular drainage. Results furthermore suggest that GnRH or hCG given at the time of insemination could reduce the ovulation failure rate of the dominant follicle following the puncture and drainage technique.

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