ABSTRACT

The 6-d timed artificial insemination protocol has been designed to advance luteolysis after the first administration of GnRH so that the preovulatory follicular diameter at second GnRH is reduced and thereby pregnancy outcome may be improved. To achieve an earlier and complete luteolysis (5 to 6 d after the first GnRH treatment), an extra PGF2α treatment must be administered to cows 24 h after the initial PGF2α treatment. Although the use of 2 PGF2α treatments increases labor costs resulting from the increased handling of cows, no alternative and efficient protocol with a single PGF2α treatment has been found to date. The objective of this study was to compare the effect of a modified 6-d synchronization protocol on the luteolytic response and final preovulatory follicle diameter. The study followed a crossover design: 14 nonlactating dairy cows were included in 2 treatment doses. All cows received a presynchronization treatment consisting of 2 administrations of a PGF2α analog (PGF) 14 d apart followed by treatment with GnRH 11 d later. After the first GnRH administration, one treatment consisted of 150 μg of d-cloprostenol 5 and 6 d later (split dose) and the other treatment consisted of 375 μg of d-cloprostenol as a single dose 6 d after the first GnRH (single large dose). All cows were then treated with a second GnRH 8 d after the first. The luteolytic response to treatment was evaluated by blood progesterone concentration and CL area regression –1 to 3 d relative to the last PGF treatment obtained by ELISA and ultrasonography, respectively. Fewer cows of the split dose tended to have complete luteolysis 3 d after the last PGF treatment compared with the cows of the single large dose (35.7 and 64.3%, respectively). The final preovulatory diameter of the dominant follicle was similar in cows from the split dose and single large dose (13.7 ± 0.3 and 13.1 ± 0.5 mm, respectively). Our results support the modification of the 6-d synchronization protocol by administering a single high dose of PGF 6 d after GnRH (with the subsequent reduction in labor resulting from reduced handling of animals) without detrimental effects on the luteolytic response of dairy cows and preovulatory diameter of the dominant follicle compared with the original protocol. However, this modification of the 6-d synchronization protocol should be tested in a large field study involving fertility data with lactating cows before its use can be recommended.

Key words: dairy cattle, luteolysis, progesterone concentration, prostaglandin F2α, dose

INTRODUCTION

Most fixed-timed AI programs involve the use of GnRH and PGF2α, or its synthetic analog (PGF), to synchronize follicle wave emergence, corpus luteum (CL) regression, and ovulation. The original Ovsynch protocol (Pursley et al., 1995) recommended the administration of PGF2α 7 d after and 2 d before the first and second GnRH treatments, respectively. A longer period of follicle dominance has been associated with lower fertility in dairy cattle. It was demonstrated that pregnant cows had a mean interval from emergence of dominant follicle to ovulation 1 d shorter than cows failing to become pregnant, which implies a smaller preovulatory follicle (Bleach et al., 2004). In addition, the preovulatory diameter of the dominant follicle at the time of final GnRH of Ovsynch is correlated with fertility (Bello et al., 2006), with better results after ovulations of small follicles. A practical way to reduce the period of dominance and the final preovulatory diameter of the dominant follicle before AI may be achieved by shortening the interval from first GnRH to PGF2α treatment and AI. However, a limiting factor to this practice is the reduced ability of the luteolytic product to regress the younger, newly formed CL. This could be partially overcome by the additional administration of a second luteolytic treatment 7 h (Kasimanickam et al., 2009) to 24 h (Santos et al., 2010) after the first PGF administration. Although this practice improved the fertility of treated cows in both studies, a limitation is the additional handling of

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1Corresponding author: juan.cuervo@uch.ceu.es
animals resulting from the administration of the extra luteolytic treatment required for full CL regression.

A recent study tested the hypothesis that reducing the number of luteolytic treatments (with the subsequent reduction in labor costs) from 2 to a single administration with a double dose of PGF would maintain fertility without decreasing the luteolytic response of cows enrolled in a 5-d timed AI protocol (Ribeiro et al., 2012). Unfortunately, the single treatment of cows with a double dose of PGF 5 d after GnRH administration induced fewer cows with full luteolysis than the original protocol consisting of 2 administrations of a standard dose of PGF 5 and 6 d after the first GnRH administration (61.7 vs. 96.2, respectively), with an equivalent reduction in pregnancies per AI.

The objective of this study was to compare the luteolytic response of cows treated with a single high dose of PGF 6 d after GnRH administration with that of cows treated with 2 administrations of a standard dose of PGF 5 and 6 d after GnRH. We hypothesized (1) that a high dose of PGF administered to nonlactating dairy cows as a single treatment 6 d after GnRH would be as efficient as 2 treatments of PGF in terms of luteolysis; and (2) that the preovulatory diameter of the dominant follicle at the time of the final GnRH administration would not be affected by treatment.

MATERIALS AND METHODS

Cows and Hormonal Treatments

This trial was conducted from May to June 2012 at the Veterinary School Research Farm of the Universidad CEU Cardenal Herrera (Náquera, Spain). All cows’ procedures were handled in accordance with the Spanish Department of Agriculture Guide for Care and Use of Animals in Research, and were approved by the Animal Welfare Committee of the Universidad CEU Cardenal Herrera.

Fourteen nonlactating Holstein cows (7 nulliparous and 7 multiparous) were used in the trial. The cows had been dry for at least 2 yr and their age varied from 2.6 to 12 yr (mean age of 5.4 ± 2.2 yr). At the beginning of the trial, all cows were cyclic and had no apparent uterine or ovarian abnormalities confirmed by ultrasonography. The cows were fed alfalfa hay and cereal concentrate ration calculated for a maintenance diet for dry cows. The mean BCS was 3.5 ± 0.6 (range 2.5 to 4, scale 1 to 5) and the mean weight was 640 ± 49 kg (range 560 to 710 kg). All the PGF (d-cloprostenol, Genestran, aniMedica GmbH, Senden, Germany) and GnRH (0.01 mg of buserelin: 2.5 mL of Buserelin, aniMedica GmbH) injections were administered with single-dose syringes in semimembranosus or semitendinosus muscles with 18-gauge 3.5-cm needles. Examination of the uterus and ovaries was performed by rectal ultrasonography with an ultrasound scanner (Sonosite 180 Vet Plus, BCF Ultrasound Australasia, Nunawading, VIC, Australia) equipped with an 8-MHz linear-array transducer.

Experimental Design

The study followed a crossover design. Each of the 14 cows was followed during 2 estrous cycles divided into 2 treatments. The 2 treatments differed in the luteolytic regimen. The split dose (n = 14) consisted of 2 administrations of 150 μg of d-cloprostenol each (2 mL of Genestran) 24 h apart; whereas the single large dose (n = 14) consisted of a single administration of 375 μg of d-cloprostenol (5 mL of Genestran). Whether a cow received one or the other luteolytic regimen during the first cycle was chosen randomly.

The rationale of choosing the single dose of 375 μg of d-cloprostenol was based on a previous study in which a slightly smaller dose (300 μg of d-cloprostenol) failed to induce full luteolysis in all cows with a single CL of 4.5 to 5 d old (Valdecarbes-Torres et al., 2012). It is worth noting that the presence of an additional older CL (presynchronized cows) may modify the sensitivity of the younger CL to a single PGF treatment (our unpublished data).

Each cow received a presynchronization treatment consisting of 2 administrations of 150 μg of d-cloprostenol 14 d apart. Eleven days later, each cow received a GnRH administration followed by 2 administration of 150 μg of d-cloprostenol 5 and 6 d later (split dose) or 375 μg of d-cloprostenol 6 d after the first GnRH (single large dose); d 0 was designated as the day on which the cows received the last PGF treatment (6 d after the first GnRH administration). Finally, a second GnRH administration was given 2 d after the last PGF treatment (Figure 1).

Ultrasound examinations of the ovaries were performed once daily (after the last PGF treatment of the presynchronization protocol) until ovulation and then again from the first GnRH administration until 2 d after the second GnRH administration. In each examination, the diameter of the 3 largest follicles, the number of ovulations, and number of CL were recorded. At the time of the luteolytic treatment, the CL were identified as primary (originating from ovulation after the last PGF treatment of the presynchronization protocol) or accessory (originating from ovulation after the first administration of GnRH). The position of each CL was mapped within the ovary for daily monitoring (from –1 to 3 d relative to the last PGF treatment) and the size expressed in area (mm²). When the CL showed a
central cavity, the area of the cavity was subtracted from the overall CL area.

Blood samples for progesterone determination were obtained –1, 0, 1, 2, and 3 d relative to the last PGF treatment in both groups for assessing the luteolytic response of cows.

**Progesterone Determination**

In both treatments, a blood sample was collected from the tail vessel on each occasion (–1 to 3 d relative to the last d-cloprostenol treatment) in 5-mL heparinized tubes. The tubes were centrifuged within 1 h for 10 min at 2,000 × g, and aliquots of plasma were stored at −20°C for later assay determination. Concentrations of plasma progesterone were measured in a single assay, using enzyme-immunoassay kits (Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany) with a sensitivity of 0.04 ng/mL and an intraassay CV of 5%.

**Statistical Analyses**

All data were tested for normality. Data not normally distributed were ranked. Sequential data (progesterone concentration and CL area) were analyzed by a general linear model of variance with a repeated statement to account for autocorrelation between sequential observations of same individuals taken at –1 d to 3 d relative to the last PGF treatment (Systat 13, Systat Software Inc., Chicago, IL). Three separate models were created to calculate the effects of treatment on the change in progesterone concentration and accessory and primary CL areas. If an effect of group, day, or group × day interaction was found, the data were further tested by unpaired Student t-test.

Frequency data (the percentage of cows with progesterone concentration <1 or 0.5 ng/mL –1 to 3 d after PGF treatment and ovulation rate) were analyzed by Fisher’s exact test. Finally, the effect of the luteolytic regimen on the diameter of the dominant follicle at the time of the final GnRH was calculated by 2-sample t-test.

**RESULTS**

The ovulatory response to the presynchronization treatment (second PGF of Presynch treatment) and to the first and second GnRH administrations did not differ significantly between treatments (92.8 and 100% ovulation rate following the Presynch; 92.8 and 78.6% after the first GnRH administration; and 100 and 100% after the second GnRH treatment, for the split and single dose treatments, respectively) or number of cycle. No cows double-ovulated after the Presynch or GnRH treatments. Luteolytic regimen had no effect on the progesterone concentration –1 to 3 d relative to treatment (Figure 2). However, we observed a treatment (luteolytic regimen) × day interaction on the progesterone concentration (P < 0.01; Figure 2). This interaction originated from an earlier decrease in the progesterone concentration in cows from the split dose on d 0 (P = 0.007; Figure 2) and from a resurgence in the progesterone concentration (P = 0.015; Figure 2) between d 2 and 3 after treatment compared with the single large dose. The rate of decrease in progesterone concentration between d 2 and 3 (0.31 ± 0.08 ng/mL) in cows treated with the single large dose was greater (P = 0.03) than that (−0.67 ± 0.27 ng/mL) in cows from the split dose at the same time points. Similarly, the rate of decrease in progesterone concentration between d 0 and 1 was greater for cows from the single large dose compared with cows from the split dose (10.1 ± 1.5 and 4.18 ± 1.3 ng/mL, respectively; P = 0.006).

The change in luteal area of the accessory CL was not influenced by luteolytic regimen (Figure 3). In both luteolytic regimens, the accessory CL area increased from d −1 to 0 and then decreased gradually until d 3. On the other hand, we found a treatment × day
interaction on the change in primary CL area, which resulted from an earlier decrease (from d –1 to 0) in the CL area of cows from the split dose compared with the single large dose (P = 0.005; Figure 3). After that point, the primary CL area of cows from both luteolytic regimens decreased gradually until d 3.

Fewer cows treated with the split dose tended (P = 0.09) to undergo full luteolysis (progesterone concentration <1 ng/mL) compared with cows treated with the single large dose (P = 0.005; Figure 3). After that point, the primary CL area of cows from both luteolytic regimens decreased gradually until d 3.

Fewer cows treated with the split dose tended (P = 0.09) to undergo full luteolysis (progesterone concentration <1 ng/mL) compared with cows treated with the single large dose on d 3 (35.7 and 64.3%, respectively; Table 1). Similarly, fewer cows (P = 0.08) treated with the split dose had their progesterone concentration decreased by 0.5 ng/mL on d 3 (14.3 and 42.3%, for the split dose and the single large dose, respectively; Table 1).

The preovulatory follicle diameter at the final administration of GnRH (d 2) did not differ significantly between treatments: 13.7 ± 0.3 and 13.1 ± 0.5 mm for the split dose and single large dose, respectively.

**DISCUSSION**

Hypothesis 1 was accepted: the difference in the number of cows with full luteolysis after treatment with either 2 doses of PGF 5 and 6 d or a single, larger dose of PGF 6 d after the first GnRH administration increased by 0.5 ng/mL on d 3 (14.3 and 42.3%, for the split dose and the single large dose, respectively; Table 1).

The preovulatory follicle diameter at the final administration of GnRH (d 2) did not differ significantly between treatments: 13.7 ± 0.3 and 13.1 ± 0.5 mm for the split dose and single large dose, respectively.

**Figure 2.** Mean ± SEM plasma progesterone concentration (P4; ng/mL) of cows from the split dose (n = 14) and the single large dose (n = 14) −1 to 3 d after the last PGF (synthetic analog of PGF2α, d-cloprostenol) treatment (6 d after the first GnRH treatment). In the split dose, cows received 2 administrations of 150 μg of d-cloprostenol 5 and 6 d after the first GnRH treatment (solid black arrows). In the single large dose, cows received a single administration of 375 μg of d-cloprostenol 6 d after the first GnRH treatment. A second GnRH was administered 2 d after the last PGF treatment (8 d after the first GnRH). All cows ovulated within 48 h of the second GnRH treatment (Ov). The probabilities for the effect of treatment (luteolytic regimen: T), day (D), and treatment by day interaction (T×D) on the progesterone concentration are shown. Asterisk indicates a significant difference between progesterone concentrations on a given day after the last PGF treatment.

**Figure 3.** Mean (± SEM) luteal area (mm) of primary and accessory corpus luteum (CL) of cows from the split dose and the single large dose −1 to 3 d after the last PGF treatment (6 d after the first GnRH treatment). In the split dose treatment, cows received 2 administrations of 150 μg d-cloprostenol 5 and 6 d (white arrow) after the first GnRH treatment. In the single large dose treatment, cows received a single administration of 375 μg of d-cloprostenol d after the first GnRH treatment. The primary CL originated from ovulations during the presynchronization protocol. The accessory CL originated from ovulations after the first GnRH treatment. The ages of the primary and accessory CL at the time of the last PGF (synthetic analog of PGF2α, d-cloprostenol) treatment were approximately 12 and 4.5 d, respectively. The probabilities for the effect of treatment (luteolytic regimen: T), day (D) and treatment by day interaction (T×D) on CL area are shown.

### Table 1. Effect of dose and timing of the luteolytic treatment on luteolysis [progesterone (P4) concentration −1 to 3 d relative to last prostaglandin F treatment]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cows with P4 &lt;1 ng/mL (%)</th>
<th>Cows with P4 &lt;0.5 ng/mL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d −1  d 0  d 1  d 2  d 3</td>
<td>d −1  d 0  d 1  d 2  d 3</td>
</tr>
<tr>
<td>Split dose</td>
<td>0.0  0.0  14.2  42.9  35.7 *</td>
<td>0.0  0.0  7.1  14.3  14.3 *</td>
</tr>
<tr>
<td>Single dose</td>
<td>0.0  0.0  7.1  50.0  64.3 b</td>
<td>0.0  0.0  0.0  14.3  42.3 b</td>
</tr>
</tbody>
</table>

*Percentages within a row with different superscripts differ (P < 0.1).

1Split dose = 2 treatments of 150 μg of d-cloprostenol administered to cows (n = 14) 5 and 6 d after GnRH treatment; single dose = a single large dose of 375 μg of d-cloprostenol administered to cows (n = 14) 6 d after GnRH treatment.
was not significant. Surprisingly, the number of cows with full luteolysis tended to be even greater with the single large dose. This is in contrast to previous reports in which the luteolysis rate of lactating dairy cows was superior following 2 treatments of PGF 5 and 6 d after GnRH administration compared with a single treatment of a standard dose (Santos et al., 2010) or a double dose (Ribeiro et al., 2012) of PGF 5 d after GnRH administration. The lack of significant difference between the number of cows with full luteolysis in the treatments in the present study might be due to the delay in the administration (1 d later) of the single high dose of PGF (6 d after GnRH) compared with the reported studies (5 d after GnRH). Another possible explanation for this lack of significance is a Type II error because of an insufficient number of cows per treatment to detect a difference.

Most cows treated with PGF 5 d after GnRH will have a CL aged 3.5 to 4 d old. It has been shown (Valldecabres-Torres et al., 2012) that with such a young CL, the luteolytic response to a single treatment of PGF (regardless of the dose) is inadequate (0 to 20% of cows with complete luteolysis). In contrast, cows with a CL 1 d older (4.5 to 5 d old) have a greater luteolytic response (20 to 80% of cows with complete luteolysis) to a single treatment of PGF. At this stage of CL maturity, the luteolytic response is dependent on the PGF dose (Valledecabres-Torres et al., 2012).

In cows treated with the split dose, the first PGF administration induced a significant decrease in progesterone concentration within 1 d of treatment. However, the decrease in progesterone concentration seemed to be accounted for by the regression of the primary CL and not of the accessory CL, which continued to grow at a similar rate as that in nontreated cows until d 0 (single large dose). The lack of sensitivity of the accessory CL (3.5 to 4 d old) in a 5-d synchronization protocol might explain the suboptimal luteolytic response when a single treatment is administered 5 d after GnRH, regardless of dose (Santos et al., 2010; Ribeiro et al., 2012).

The different physiological state of cows from the reported (lactating cows) and present study (nonlactating cows) might also account for differences in the luteolytic response. In the reported studies, a single treatment of PGF 5 d after GnRH (regardless of dose) induced full luteolysis in approximately 60% of lactating cows compared with >90% when an additional treatment of PGF was administered 24 h later (Santos et al., 2010; Ribeiro et al., 2012). The overall luteolytic responses in the reported studies seem superior to those observed in the present study (<60% in both groups). Furthermore, the different techniques of assaying progesterone between the reported and present studies (RIA and ELISA, respectively) might account for such a difference (Colazo et al., 2008).

Finally, it is not known whether a smaller dose of PGF 6 d after GnRH would induce a similar luteolytic response to that observed in cows receiving the single large dose (375 μg of d-cloprostenol). Further research should be carried out to test this hypothesis.

Hypothesis 2 was also accepted: the preovulatory diameter of the dominant follicle at the final GnRH administration did not differ between groups. This is relevant to the dairy industry because the diameter of the dominant follicle has been correlated with fertility (Bello et al., 2006). In addition, the main purpose of a 5-d synchronization protocol is to reduce the period of follicular dominance (Santos et al., 2010). The lack of difference in follicle diameter between cows of both groups is not surprising because the interval between the first and final GnRH administrations remained constant (8 d), as did the interval between the last PGF and the final GnRH treatments (2 d).

**CONCLUSIONS**

The results of the present study support the modification of the 6-d synchronization protocol by administering a single high dose of PGF 6 d after GnRH (with the subsequent reduction in labor resulting from reduced handling of cows) without detrimental effects on the luteolytic response of dairy cows and preovulatory diameter of the dominant follicle, compared with the original protocol. However, this modification of the 6-d synchronization protocol should be tested in a large field study involving fertility data with lactating cows before its use can be recommended.

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