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Effect of human chorionic gonadotrophin administration 2 days after insemination on progesterone concentration and pregnancy per artificial insemination in lactating dairy cows

J. M. Sánchez,* F. Randi,* C. Passaro,* D. J. Mathew,* S. T. Butler,† and P. Lonergan*[1]
*School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland, D04N2E5
†Animal Research Department, Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark, Fermoy, County Cork, Ireland, P61C996

ABSTRACT

The aim of this study was to examine the effect of a single administration of human chorionic gonadotrophin (hCG) during the establishment of the corpus luteum (CL) on progesterone (P4) concentration and pregnancy per artificial insemination (P/AI) in lactating dairy cows. Postpartum spring-calving lactating dairy cows (n = 800; mean ± SD days in milk and parity were 78.5 ± 16.7 and 2.3 ± 0.8, respectively) on 3 farms were enrolled on the study. All cows underwent the same fixed-time AI (FTAI) protocol involving a 7-d progesterone-releasing intravaginal device with gonadotrophin-releasing hormone (GnRH) administration at device insertion, prostaglandin at device removal followed by GnRH 56 h later, and AI 16 h after the second GnRH injection. Cows were blocked on days postpartum, body condition score, and parity and randomly assigned to receive either 3,000 IU of hCG 2 d after FTAI or no further treatment (control). Blood samples were collected on d 7 and 14 postestrus by coccygeal venipuncture on a subset of 204 cows to measure serum P4 concentration, and pregnancy was diagnosed by ultrasonography approximately 30 and 70 d after FTAI. Administration of hCG caused an increase in circulating P4 concentrations compared with the control treatment on d 7 (+22.2%) and d 14 (+25.7%). The P/AI at 30 d after FTAI was affected by treatment, farm, body condition score, and calving to service interval. Overall, administration of hCG decreased P/AI (46.3% vs. 55.1% for the control). Among cows that did not become pregnant following AI, a greater proportion of control cows exhibited a short repeat interval (≤17 d) compared with cows treated with hCG (8.6% vs. 2.8%, respectively). In addition, the percentages of cows pregnant at d 21 (59.6% vs. 52.0%) and d 42 (78.3% vs. 71.9%) were greater in control than in hCG-treated cows. The overall incidence of embryo loss was 10.7% and was not affected by treatment. There was a tendency for an interaction between treatment and CL status at synchronization protocol initiation for both P4 concentration and P/AI. In conclusion, administration of hCG 2 d after FTAI increased circulating P4 concentrations. Unexpectedly, cows treated with hCG had lower fertility; however, this negative effect on fertility was manifested primarily in cows lacking a CL at the onset of the synchronization protocol.

Key words: human chorionic gonadotrophin, progesterone, timed artificial insemination, pregnancy per artificial insemination

INTRODUCTION

A fundamental goal in seasonal-calving pasture-based systems of dairy production is to achieve a 12-mo calving interval to synchronize grass growth with herd dietary demand (Dillon et al., 1995). Pregnancy loss is one of the major causes of reproductive failure in cattle and leads to extended calving intervals. In a seasonal system, this can have a significant effect on profitability (Shalloo et al., 2014). Despite high fertilization rates (80–90%), many studies have reported that only 30% or less of high-producing lactating cows calve after a single AI (Wiltbank et al., 2016). Most of the loss occurs before maternal recognition of pregnancy, in the period between fertilization and d 16 after insemination (Diskin and Morris, 2008), with a significant proportion occurring before d 7 in high-producing dairy cows (Sartori et al., 2010).

The steroid hormone progesterone (**P4**) plays a key role in the reproductive events associated with the establishment and maintenance of pregnancy (reviewed by Lonergan et al., 2016, and Spencer et al., 2016). Low circulating P4 concentrations in the early postovulatory period have been reported in several studies to have an adverse effect on conceptus elongation (Mann and

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[1]Corresponding author: pat.lonergan@ucd.ie

Lamming, 2001; Forde et al., 2011) and the endometrial transcriptome (Forde et al., 2012) and are ultimately associated with reduced fertility in lactating dairy cows (Diskin et al., 2006; Wiltbank et al., 2014). On the other hand, elevated concentrations of circulating P4 in the period immediately after conception have been associated with advanced conceptus elongation (Carter et al., 2008), increased interferon-tau production (Mann and Lamming, 2001; Rizos et al., 2012), and greater pregnancy rates in cattle (Stronge et al., 2005; McNeill et al., 2006) and sheep (Ashworth et al., 1989).

In cattle, human chorionic gonadotrophin (**hCG**) has luteinizing hormone (**LH**)-like activity but has a longer half-life than LH. Administration of hCG has been reported to (1) prolong the life span of the corpus luteum (**CL**; Sianangama and Rajamahendran, 1992), (2) induce ovulation throughout the estrous cycle (Santos et al., 2001), (3) stimulate the emergence of accessory CL (Rajamahendran and Sianangama, 1992), and (4) alter follicular wave dynamics (Diaz et al., 1998). Administration of hCG increases endogenous P4 produced by the CL (for review, see De Rensis et al., 2010; Lonergan, 2011). Typically, hCG is administered around d 5 postestrus to coincide with the first wave dominant follicle, resulting in ovulation and formation of an accessory CL, leading to increased circulating P4 concentrations (Santos et al., 2001; Nascimento et al., 2013b). In addition to forming an accessory CL, hCG administration can lead to hypertrophy of the original CL (Rizos et al., 2012).

Despite its positive effects on circulating P4, the effect of hCG administration on pregnancy rates has been inconsistent (Lonergan, 2011; Wiltbank et al., 2014). An elevation in circulating P4 concentrations occurs 3 d after hCG injection when it is administered on d 5 after AI (Rajamahendran and Sianangama, 1992; Santos et al., 2001; Stevenson et al., 2007; Vasconcelos et al., 2011). In a recent large study, Nascimento et al. (2013a) reported a modest increase of 3% (meta-analysis study; n = 4,397 cows) or 3.5% (manipulative study; n = 2,979 cows) in pregnancy rate following hCG administration 5 d after AI. A beneficial effect on pregnancy per AI has been reported in repeat-breeding dairy cows treated with hCG 6 d after AI (Alnimer and Shamoun, 2015). An early increase in P4 has been associated with improved pregnancy rates in cattle; however, administration of hCG on d 5 to 7 does not affect circulating P4 for several days, partly due to the time required for ovulation and accessory CL formation (Rizos et al., 2012). We previously evaluated the effect of a single administration of hCG on d 1, 2, 3, or 4 after estrus on P4 concentrations in beef heifers and observed that administration of hCG on d 2 resulted in increased CL area from d 6 to 12 and increased P4 in circulation from d 6 (Maillo et al., 2014).

Therefore, the objective of this study was to investigate the effect of hCG administration 2 d after fixed-time AI (**FTAI**) on P4 concentrations and pregnancy per AI (**P/AI**) in lactating dairy cows managed under pasture-based systems. We hypothesized that hCG treatment administered on d 2 after an FTAI program would increase circulating concentrations of P4 during the early luteal phase and thereby enhance fertility in lactating dairy cows.

MATERIALS AND METHODS

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with Statutory Instrument No. 543 of 2012 (under Directive 2010/63/EU on the protection of animals used for scientific purposes).

Animals and Experimental Design

Lactating Holstein Friesian dairy cows (n = 846) located on 3 commercial spring-calving farms in Ireland were enrolled in this study (78.5 ± 16.7 DIM) conducted between April and August 2016. The average parity was 2.3 ± 0.80 (mean ± SD). Cows that were ≥35 DIM were deemed eligible for enrollment in the study. Estrous cycles were synchronized using a 7-d P4-releasing intravaginal device (**PRID**; containing 1.55 g of P4; PRID E, Ceva Sante Animale, Libourne, France), with administration of $PGF_{2\alpha}$ (Enzaprost, 5 mL equivalent to 25 mg of dinoprost; Ceva Sante Animale) at device removal. Gonadotrophin-releasing hormone containing 0.1 mg gonadorelin diacetate (Ovarelin, Ceva Sante Animale) was administered at PRID insertion (d −10) and at 56 h after PRID removal (d −1) to stimulate ovulation of the dominant follicle. Cows were inseminated 72 h after PRID removal (i.e., 16 h after the second GnRH). Body condition score was recorded at PRID insertion by the same 2 people using a scale from 1 (emaciated) to 5 (fat) according to Ferguson et al. (1994). On d 2 after FTAI (d 0), cows were blocked on parity, DIM, and BCS and randomly allocated to receive 3,000 IU of hCG (Chorulon; Intervet, Dublin, Ireland) by intramuscular injection or no further treatment (Figure 1).

Blood Sampling and P4 Assay

Blood samples were collected by coccygeal venipuncture from a subset of cows (n = 204) from both treatments on d 7 and 14 post-FTAI to measure serum P4

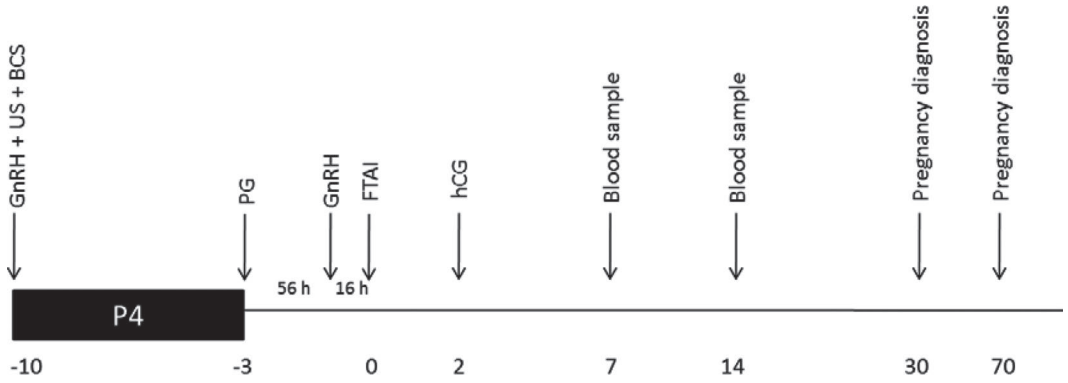

Figure 1. Experimental design. Pregnancy diagnosis was carried out by ultrasound scanning (US) at both 30 and 70 d after fixed-time AI. P4 = intravaginal progesterone device; PG = prostaglandin; FTAI = fixed-timed AI; hCG = human chorionic gonadotrophin.

concentrations. Blood samples were stored at 4°C for 12 to 24 h before being centrifuged at 1,500 × *g* at 4°C for 20 min. Using a Pasteur pipette, serum was separated and stored at −20°C until analysis of P4 concentrations by solid-phase RIA using a PROG-RIA-CT kit (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium) according to the manufacturer's instructions. The sensitivity of the assay was 0.05 ng/mL. The interassay coefficients of variation for quality control samples were 4.79% (low), 3.55% (medium), and 2.42% (high). The intra-assay coefficients of variation were 9.93% (low), 8.07% (medium), and 6.13% (high).

Reproductive Measurements

The following reproductive measurements were calculated and analyzed: calving to first service interval (**CSI**; interval in days from calving to first service), mating start date to conception interval (interval in days from mating start to conception determined by subsequent pregnancy detection), P/AI at first service (**P/AI1**; percentage of cows confirmed pregnant by ultrasonography 30 d after FTAI), conception rate at second service (**P/AI2**; percentage of cows confirmed pregnant by ultrasonography after second AI), repeat interval (**RI**; interval in days from FTAI to second AI), embryo loss rate (**EL**; percentage loss of a viable pregnancy between diagnosis at 30 d and 70 d after FTAI), and 21- and 42-d pregnancy rate (**PR21** and **PR42**; percentage of cows that successfully established pregnancy during the first 21 and 42 d of the breeding season, respectively).

The reproductive tracts of all animals were examined via transrectal ultrasound immediately before initiation of the synchronization protocol using a portable ultrasound machine (Easi-Scan; BCF Technology Ltd., Bellshill, Scotland) fitted with a 4.5- to 8.5-MHz linear array transducer. The number of CL and dominant follicles was recorded for all cows. Cows with uterine disease were not enrolled in the study. To determine P/AI1, P/AI2, PR21, PR42, and EL, all synchronized cows were scanned at 28 to 32 d (30 d) and 66 to 74 d (70 d) after FTAI (Figure 1). Embryo loss was calculated as follows:

$$\text{EL (\%)} = (\text{no. of pregnant cows at 30 d} - \text{no. of pregnant cows at 70 d/no. of pregnant cows at 30 d}) \times 100.$$

Compliance to Protocol

On the first day of the study, 846 cows were scanned by transrectal ultrasound. Any cows exhibiting ovarian or uterine pathology were removed from the study (n = 20). Noncompliance with the protocol, including errors in PRID removal, administration of injections, or during the insemination procedure as well as missed injections and PRID loss, were removed from the data set. Also, any cows that had a missing value for ovarian structures at the initial ultrasound scan, BCS, and pregnancy diagnosis were removed from the data set. Finally, data generated from 799 cows were used for statistical analysis.

Statistical Analysis

The effect of the independent variables on all binary variables was determined using a generalized linear mixed model (GLIMMIX) in SAS (SAS Institute Inc., Cary, NC). Fixed effects in the model included treatment (control or hCG), farm (A, B, or C), presence of CL at initiation of the synchronization protocol (0, 1), presence of dominant follicle at initiation of the syn-

chronization protocol (0, 1), BCS (≤2.5 and ≥2.75), parity (1, 2, and ≥3), and CSI (<60, 60–80, and >80 d). Cow was included as a random effect. A logit link function was used and a binary response distribution was specified. All effects and 2-way interactions were tested in each model and manually removed by backward elimination if nonsignificant ($P > 0.05$). The only exceptions were treatment, farm, parity, BCS, and CSI, which were forced into all models. Survival analysis was carried out using the LIFETEST procedure of SAS to examine the effect of treatment on mating start date to conception interval.

The effect of treatment on P4 concentrations was determined using mixed models (MIXED) in SAS. The effect of treatment was evaluated for different categories of cows: (1) all cows, (2) cows that were pregnant on d 30, (3) cows that were not pregnant on d 30, and (4) cows that were not pregnant on d 30 and did not have a short RI. Progesterone concentration data were transformed to generate a normal distribution, and extreme outliers were removed (n = 4), resulting in a final P4 concentration data set of 404 records (n = 201 on d 7; n = 203 on d 14).

RESULTS

Pregnancy/AI and RI

The P/AI1 was affected by treatment ($P = 0.02$), farm ($P < 0.001$), BCS ($P = 0.03$), and CSI ($P = 0.04$), and there was a tendency for an interaction between treatment and CL status at synchronization protocol initiation ($P = 0.07$). The main results are summarized in Table 1. Overall, administration of hCG decreased ($P = 0.02$) P/AI1 by 8.8 percentage points. The P/AI for cows with BCS ≤2.5 at the beginning of the synchronization protocol was 9.4 percentage points less than that for cows with BCS ≥2.75. The P/AI1 was less for cows inseminated at <60 d postpartum compared with cows inseminated between 60 and 80 d (−13.2 percentage points) and >80 d postpartum (−11.7 percentage points). The P/AI1 was similar in control cows with or without a CL on the ovary at the time of protocol initiation. Conversely, cows administered hCG that did not have a CL on the ovary at protocol initiation had a 12.4% point reduction in P/AI1 compared with cows administered hCG that did have a CL on the ovary at protocol initiation (Figure 2).

Among cows that did not become pregnant following FTAI, a greater ($P < 0.05$) proportion of control cows exhibited short RI (≤17 d; 8.6%; 14/163) compared with cows treated with hCG (2.8%; 5/179). No differences between treatments were observed for the percentage of cows exhibiting either normal (18–24 d) or prolonged (≥25 d) RI. There was no effect of treatment on P/AI2 (57.9% vs. 58.2% for control and hCG, respectively; $P = 0.95$).

Embryo Loss

The overall incidence of embryo loss was 10.7% and was not affected by treatment ($P = 0.38$) or parity ($P = 0.16$). There was a tendency ($P = 0.07$) for greater embryo loss in both primiparous cows (+9.6 percentage points) and parity ≥3 cows (+8.0 percentage points) compared with parity 2 cows (Table 2). Cows inseminated at <60 d after calving had greater embryo loss than cows inseminated at 60 to 80 d (+6.9 percentage points) or >80 d (+10.9 percentage points; Table 2).

21-d and 42-d Pregnancy Rate

The percentage of cows pregnant at 21 and 42 d after mating start date tended to be greater in the control than in the hCG treatment (see Table 3). No difference was detected in PR21 between cows inseminated be-

Table 1. Effect of treatment (control or human chorionic gonadotrophin, hCG), farm, parity, corpus luteum (CL) status at initiation of synchronization protocol, BCS, and calving–service interval (CSI) on percentage of cows pregnant at 30 d after fixed-time AI in lactating dairy cows

Variable	CR1[1] [% (no./total)]	*P*-value
Treatment		
Control	55.1[a] (234/408)	0.02
hCG	46.3[b] (200/391)	0.02
Farm		
A	62.8[x] (213/342)	<0.001
B	48.2[y] (157/306)	<0.001
C	41.0[y] (64/151)	<0.001
Parity		
1	48.2 (106/199)	0.64
2	53.5 (87/155)	0.64
≥3	50.4 (241/445)	0.64
CL status		
Absent	48.0 (128/259)	0.19
Present	53.3 (306/540)	0.19
BCS		
≤2.50	46.0[a] (267/494)	0.03
≥2.75	55.4[b] (167/305)	0.03
CSI		
<60 d	42.4[a] (62/144)	0.04
60–80 d	55.6[b] (137/239)	0.04
>80 d	54.1[b] (235/416)	0.04

[a,b]Within a variable, values with different superscripts differ ($P < 0.05$).

[x,y]Within a variable, values with different superscripts differ ($P < 0.01$).

[1]Conception rate at 30 d after first fixed-time AI is reported as the model-adjusted least squares means (%) and the unadjusted number of animals that successfully conceived (no./total).

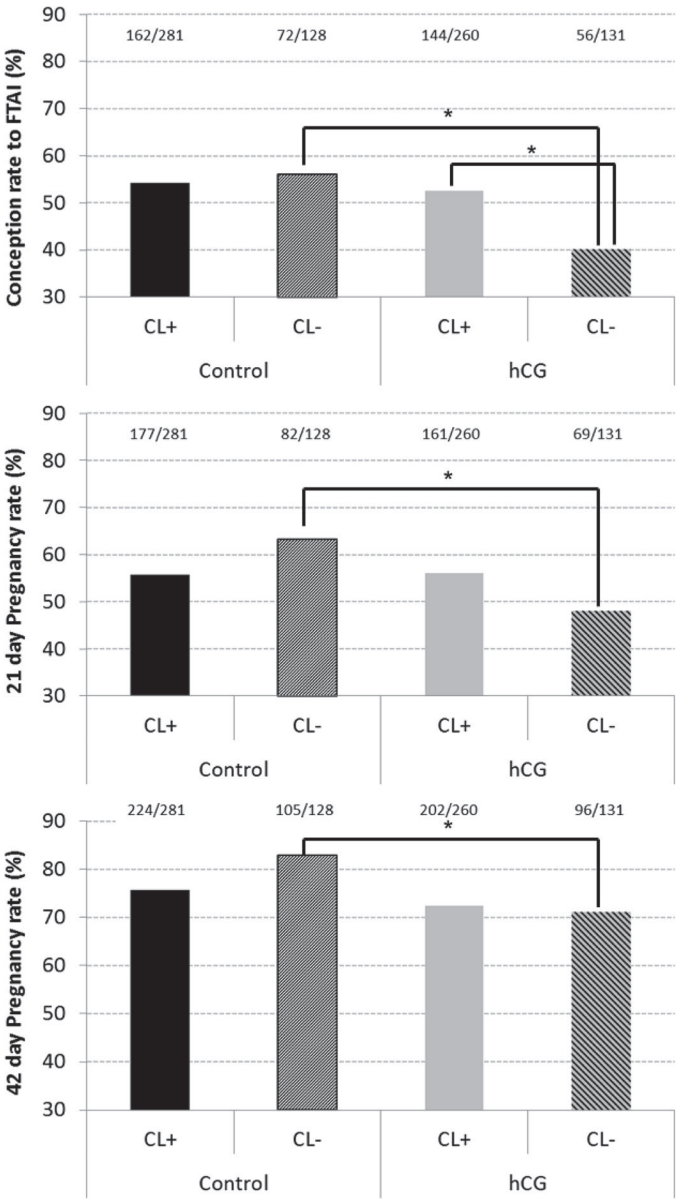

Figure 2. Interaction between treatment [control or human chorionic gonadotrophin (hCG)] and corpus luteum (CL) status for pregnancy per AI at first service (top panel; $P = 0.07$), 21-d pregnancy rate (middle panel; $P = 0.05$), and 42-d pregnancy rate (bottom panel; $P = 0.2$). Within each panel, significant differences ($P < 0.05$) are indicated by an asterisk (*). The values above each column indicate the number of animals for each outcome variable (unadjusted). FTAI = fixed-time AI.

tween 60 and 80 d postpartum and >80 d postpartum, but both groups had a greater PR21 than cows inseminated <60 d after calving. In contrast, all 3 calving interval groups differed ($P < 0.01$) from each other for PR42; cows inseminated >80 d postpartum had the greatest PR42, cows inseminated <60 d had the lowest

Table 2. Effect of treatment (control or human chorionic gonadotrophin, hCG), farm, parity, and calving–service interval (CSI) on embryo loss rate (EL) in lactating dairy cows

Variable	EL [% (no./total)]	*P*-value
Treatment		
Control	9.9 (22/233)	0.38
hCG	12.8 (24/196)	0.38
Farm		
A	11.2^{ab} (23/212)	0.01
B	5.9^{a} (9/157)	0.01
C	20.7^{b} (14/60)	0.01
Parity		
1	15.8^{x} (13/105)	0.16
2	6.2^{y} (5/85)	0.16
≥3	14.2^{x} (28/239)	0.16
CSI		
<60 d	17.9^{a} (13/61)	0.05
60–80 d	11.0^{ab} (17/135)	0.05
>80 d	7.0^{b} (16/233)	0.05

^{a,b}Within a variable, EL with different superscripts differ ($P \leq 0.01$).
^{x,y}Within a variable, EL with different superscripts tends to differ ($P = 0.07$).

PR42, and cows that were 60 to 80 d postcalving were intermediate (Table 3).

Presence of a CL at the beginning of the synchronization protocol was associated with greater PR21 ($P = 0.05$). Presumed acyclic cows (i.e., lacking a CL) treated with hCG had a lower PR21 and PR42 than acyclic control cows (−15.2 and −11.7 percentage points, respectively; Figure 2). No differences in PR21 or PR42 were found between cyclic hCG-treated and cyclic control cows.

An interaction between treatment and farm was also observed for PR42. There was no difference between the control and hCG treatments for PR42 on either farm A (79.7 vs. 80.7%; $P = 0.83$) or farm B (83.6 vs. 78.7%; $P = 0.33$), but PR42 was greater for the control treatment compared with the hCG treatment on farm C (74.3 vs. 51.4%, respectively; $P < 0.01$).

Survival Curve

The reproductive performance of both treatments is illustrated in Figure 3. As FTAI was used in the study, submission rate was 100% on the first day of the breeding season (i.e., mating start date). The difference between the 2 treatments in the proportion of nonpregnant cows during the breeding season reflected differences in P/AI1, as P/AI when cows returned to estrus was similar in both treatments. The differences between treatments were maintained during the first 6 wk of the breeding season, with the hCG treatment having a greater proportion of nonpregnant cows (+6.4 percentage points) after 42 d of breeding.

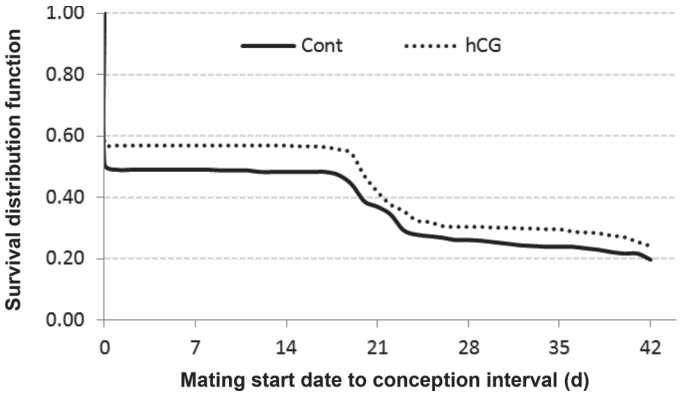

Figure 3. Survival distribution function for the interval in days from mating start date to conception for lactating dairy cows treated with 3,000 IU of human chorionic gonadotrophin (hCG treatment) or not (control treatment; Cont) on d 2 postinsemination. The y-axis is the proportion of animals not pregnant.

Concentration of Serum P4

Administration of hCG increased serum P4 concentrations compared with control cows on d 7 (+22.2%; $P = 0.02$) and d 14 (+25.7%; $P < 0.001$; Figure 4). Cows that were diagnosed pregnant on d 30 that had been treated with hCG had greater circulating P4 concentrations on d 7 [6.81 (5.99–7.68) vs. 5.24 (4.55–5.97) ng/mL; $P < 0.01$] and on d 14 [14.39 (13.07–15.79) vs. 12.01 (10.85–13.24); $P < 0.01$] compared with control cows (back-transformed LSM with 95% CI in parentheses). In cows that were diagnosed nonpregnant on d 30, circulating P4 concentrations on d 7 did not differ between treatments [4.05 (2.88–5.47) vs. 4.42 (3.18–5.91) ng/mL; $P = 0.52$], but hCG administration did cause greater circulating P4 concentrations on d 14 [7.98 (6.18–10.07) vs. 10.70 (8.52–13.19); $P = 0.02$].

Cows that had a visible CL at the time of synchronization protocol initiation and that were treated with hCG had greater circulating P4 concentrations on d 7 ($P < 0.01$) and on d 14 ($P < 0.001$) compared with control cows (Figure 5A). In cows that did not have a visible CL at the time of synchronization protocol initiation, however, circulating P4 concentrations did not differ between treatments (Figure 5B).

DISCUSSION

The main finding of this study was that despite stimulating an elevation in circulating P4 concentrations at d 7 and 14 post-AI, administration of hCG 2 d after insemination was not associated with an improvement in reproductive performance. Indeed, hCG administration had a detrimental effect on P/AI1.

Most studies that have examined the effect of hCG in cattle have administered it around d 5 postestrus to induce ovulation of the first wave dominant follicle, formation of an accessory CL, and an associated elevation in circulating P4 concentrations (Santos et al., 2001). Circulating P4 concentrations usually do not increase substantially above control levels for several days after hCG administration (Kerbler et al., 1997; Rizos et al., 2012) due to the time required for ovulation and CL formation. The results of a previous study from our group (Maillo et al., 2014) provided evidence that administration of a single injection of hCG to beef heifers on d 2, 3, or 4 after estrus stimulated enhanced development of the original CL, resulting in increased P4 concentrations. Although based on a small number of beef heifers, these observations prompted us to hypothesize that administration of hCG on d 2 postestrus would have a similar effect in lactating dairy cows and

Table 3. Effect of treatment (control or human chorionic gonadotrophin, hCG), parity, and calving–service interval (CSI) on percentage of lactating cows pregnant at 21 and 42 d after mating start date (PR21 and PR42, respectively) following fixed-time AI

Variable	PR21 [% (no./total)]	*P*-value	PR42 [% (no./total)]	*P*-value
Treatment				
Control	59.6 (259/409)	0.06	78.3 (329/409)	0.06
hCG	52.1 (230/391)		72.0 (298/391)	
Farm				
A	63.8[a] (229/342)		79.7[c] (280/342)	
B	59.6[a] (195/306)	<0.001	80.4[c] (255/306)	<0.001
C	43.9[b] (65/152)		63.8[d] (92/152)	
CSI				
<60 d	41.1[a] (58/145)		63.1[e] (88/145)	
60–80 d	61.1[b] (149/239)	<0.001	76.3[f] (183/239)	<0.001
>80 d	65.0[b] (282/416)		83.7[g] (356/416)	

[a,b]Within a variable, PR21 with different superscripts differ ($P < 0.01$).

[c,d]Within farm, PR42 with different superscripts differ ($P < 0.01$).

[e–g]Within CSI, PR42 with different superscripts differ ($P \leq 0.03$).

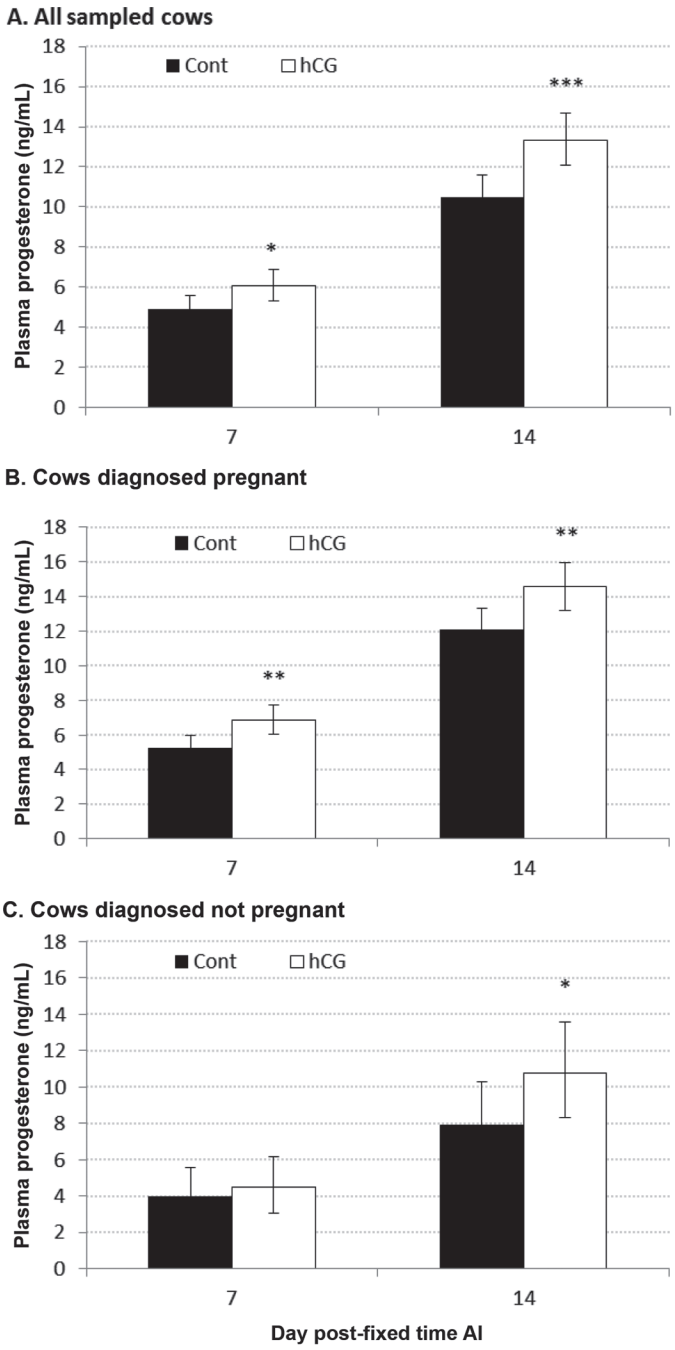

Figure 4. Progesterone concentrations on d 7 and 14 in lactating dairy cows administered 3,000 IU of human chorionic gonadotrophin on d 2 postinsemination (hCG treatment) or not (control treatment; Cont). (A) All cows that had blood samples collected (n = 204 cows; treatment effect, $P < 0.001$; treatment × day effect, $P = 0.20$). (B) Cows that had blood samples collected and that were pregnant on d 30 (n = 120 cows; treatment effect, $P = 0.001$; treatment × day effect, $P = 0.94$). (C) Cows that had blood samples collected and that were not pregnant on d 30 (n = 84 cows; treatment effect, $P = 0.10$; treatment × day effect, $P = 0.07$). Treatments differed at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ significance levels. The error bars represent the 95% CI of the back-transformed LSM progesterone concentration.

that this would be associated with an increased likelihood of pregnancy establishment.

The role of P4 in uterine receptivity is unequivocal (Forde et al., 2009; reviewed by Lonergan et al., 2016; Spencer et al., 2016). Low circulating P4 results in underdeveloped conceptuses (Forde et al., 2012) with an altered transcriptomic signature (Barnwell et al., 2016) and a low likelihood of establishing a pregnancy (Stronge et al., 2005; McNeill et al., 2006). Elevated P4 results in an acceleration of conceptus elongation (Carter et al., 2008; O'Hara et al., 2014a,b) and increased interferon-tau production (Rizos et al., 2012). Interestingly, these advanced conceptuses (Garrett et

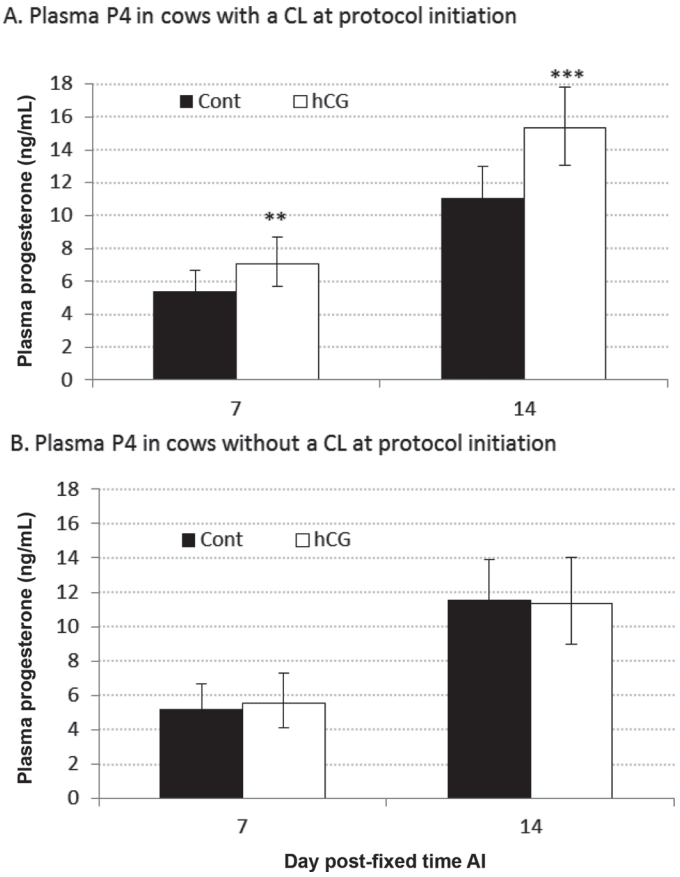

Figure 5. Effect of corpus luteum (CL) status at protocol initiation on circulating progesterone (P4) concentrations on d 7 and 14 in lactating dairy cows administered 3,000 IU of human chorionic gonadotrophin on d 2 postinsemination (hCG treatment) or not (control treatment; Cont). (A) Cows that had a visible CL at the time of synchronization protocol initiation (n = 154 cows; treatment effect, $P < 0.001$; treatment × day effect, $P = 0.09$). (B) Cows that did not have a visible CL at the time of synchronization protocol initiation (n = 50 cows; treatment effect, $P = 0.88$; treatment × day effect, $P = 0.57$). Treatments differed at $^{**}P < 0.01$ and $^{***}P < 0.001$ significance levels. The error bars represent the 95% CI of the back-transformed LSM progesterone concentration.

al., 1988; Ledgard et al., 2012) do not consistently achieve improved pregnancy rates (Randi et al., 2016). Although the mechanism has not been explored, this may be due to asynchrony between trophoblast development and the development of the embryonic disk (Degrelle et al., 2012).

In cattle, elevated P4 concentrations can be achieved by supplying exogenous P4 (intravaginal device or injection), by inducing the ovulation of a dominant follicle and the emergence of an accessory CL, or by promoting changes in an existing CL that lead to an increase in its steroidogenic capacity. Provision of exogenous P4 can be problematic, especially if the P4 is administered early in the luteal phase, with several studies reporting early regression of the CL and a short cycle (Ginther, 1970; Burke et al., 1994; O'Hara et al., 2014a; Randi et al., 2016). Wiltbank et al. (2014) emphasized that the primary factor determining greater P4 production is likely to be CL volume and the number of large luteal cells due the constitutive nature of P4 production in the bovine CL. Therefore, the use of luteotrophic agents such as hCG may be more beneficial to elevate peripheral concentrations of P4 than the direct administration of exogenous P4. The vast majority of accessory CL induced by hCG, however, are smaller and persist for a shorter period of time compared with the original CL (Sianangama and Rajamahendran, 1996; Stevenson et al., 2008b). In addition, if the accessory CL (induced by treatment with GnRH) is on the ovary contralateral to the original CL, the majority regress during the second month of pregnancy (Baez et al., 2017).

A previous study from our group (Maillo et al., 2014) concluded that administration of hCG on d 2 postestrus to beef heifers increased CL area from d 6 to 12 and increased circulating P4 concentrations from d 6 to 11 compared with control heifers. Also, increased circulating P4 concentrations were reported in recipient beef heifers injected with hCG on d 2 (5 d before embryo transfer) by O'Hara et al. (2014b). de Souza (2015) reported that treating lactating dairy cows (n = 19) with hCG 3 d after synchronized ovulation resulted in greater CL volume from d 9 to 13 and greater P4 concentrations on d 9 (6 d after treatment) compared with untreated cows. Conversely, administering hCG on d 5 postestrus did not cause an increase in the size of the primary CL (Santos et al., 2001; Nascimento et al., 2013a). Hence, the timing of hCG administration affects the CL response. Our results support the hypothesis that administration of hCG during early CL development promotes greater steroidogenesis and earlier elevation of circulating P4 concentrations in lactating dairy cows. Given the unlikely presence of a dominant follicle as early as 2 d after FTAI in dairy cows (Lucy et al., 1992), the greater peripheral P4 concentrations in hCG-treated cows in this study on d 7 and 14 were likely due to enhanced development of the primary CL.

Given the many physiological and pathological differences between primiparous and multiparous lactating dairy cows (Bamber et al., 2009; Dubuc et al., 2012), surprisingly, no effect of parity on P/AI1 was observed. No significant interaction between treatment and parity was found in this study. Kendall et al. (2009) reported greater conception rate in multiparous repeat-breeder cows treated with hCG on d 5 (56%) compared with controls (36%) but no effect in primiparous cows. Similarly, Sanchez et al. (2015) reported an increase in P/AI in multiparous cows only, following treatment with hCG 2 d after synchronized ovulation. Moreover, in that study, treatment with hCG 5 d after synchronized ovulation increased fertility in first-parity cows only, in agreement with Nascimento et al. (2013a).

Overall, hCG administration resulted in a decrease in P/AI1 compared with the control treatment. The detrimental effect of treatment was unexpected. There are several potential explanations: (1) P4 concentrations that were increased above optimal, with consequences for the timing of P4 receptor downregulation in the uterine luminal epithelium (Okumu et al., 2010); (2) premature luteolysis in some cows (Baez et al., 2017); or (3) asynchrony between the developing conceptus and the uterine environment or between trophoblast development and the development of the embryonic disk associated with elevated P4 (Degrelle et al., 2012; Randi et al., 2016). The decrease in P/AI1 in hCG-treated cows in our study is unlikely to be explained by premature luteolysis, however, because of a lack of difference in the percentage of hCG-treated and control cows expressing normal RI. Parr et al. (2014) and King et al. (2013) reported that elevation of peripheral P4 concentrations in cows with no a priori deficit, as perhaps under our experimental conditions, could result in an excess of P4, perhaps causing an asynchrony between the embryo and uterus. This could have negative consequences for the successful establishment of pregnancy (Pope, 1988; Randi et al., 2016) and may partially explain the unfavorable effect of treatment in the current study.

In several large studies that reported increased fertility following administration of hCG on d 5, the effect was frequently confined to a small number of farms (Stevenson et al., 2007; Nascimento et al., 2013a). In many studies that administered hCG approximately 5 d after AI, despite a positive effect on circulating P4 concentrations, the effect on pregnancy rates was inconsistent (Lonergan, 2011; Wiltbank et al., 2014). In one of the few other studies (de Souza, 2015) evaluating the effect of hCG administration early after FTAI (d 3; d 0 = FTAI) on conception rates in lactating dairy cows

(n ≈ 250/treatment), no treatment effect was reported. In agreement with our observations, Parr et al. (2014) reported that exogenous P4 supplementation (using an intravaginal device) from d 4 to 9 after estrus had a negative effect on pregnancy rate (control = 56% vs. 44%). King et al. (2013) observed a differential effect of hCG (on d 4–6; no effect of day of treatment) depending on P4 concentrations at treatment application. In dairy heifers with low initial plasma P4 concentrations (<2 ng/mL), no effect of hCG was noted. In heifers with high initial P4 concentrations (>2 ng/mL), however, pregnancy rate was lower compared with untreated animals (51% vs. 64%, respectively; $P = 0.02$). In another trial conducted on repeat-breeder dairy cows, Kendall et al. (2009) concluded that the effect of hCG (on d 5) was related to initial milk P4 concentration, with no response observed in cows with very low (<2 ng/mL) or high (>4 ng/mL) P4 concentrations. However, hCG-treated cows that had medium P4 concentrations (2–4 ng/mL) had a higher pregnancy rate than control cows (53.8% vs. 41.6%). This is consistent with the quadratic relationship between P4 concentrations in the first week after AI and pregnancy rate observed in beef heifers (Diskin et al., 2006) and dairy cows (Stronge et al., 2005).

There was no overall effect of CL status at synchronization protocol initiation on conception rate at 30 d after first fixed-time AI (**CR1**). These findings are in agreement with other studies. For example, Stevenson et al. (2008a) reported that supplementation with P4 (via an intravaginal device) to cows without a CL increased pregnancies per AI at both 33 and 61 d after FTAI but did not differ from cows that had a CL present at the time of the first GnRH injection of Ovsynch. Similarly, Bisinotto et al. (2015) concluded that supplementation of P4 increased circulating P4 concentrations during development of the ovulatory follicle, which re-established fertility in cows lacking a CL to a level similar to that of cows in diestrus.

An interaction between treatment and CL status at protocol initiation on CR1 was observed (Figure 2). Cows administered hCG that did not have a CL on the ovary at protocol initiation had a reduced CR1 compared with cows administered hCG that had a CL on the ovary at protocol initiation. Ovulatory follicle size is greater if the follicle develops in an environment with low circulating P4 concentrations (Fricke et al., 2016). Although not measured in the current study due to logistical constraints, our first hypothesis to explain these intriguing results was that the ovulatory follicle size was larger in cows that did not have a CL at protocol initiation, which in turn resulted in a larger CL after ovulation. In this scenario, hCG treatment may have stimulated a premature and excessive increase in P4 concentrations, causing a detrimental effect on P/AI1. In the current study, however, circulating P4 concentrations did not differ between hCG-treated or control cows that did not have a visible CL at the time of synchronization protocol initiation (Figure 5B). Thus, the hypothesis above seems unlikely. The effect of hCG on CL development, as described above, has been well documented. However, its functions in other than their "traditional" target sites are still not well known. Luteinizing hormone receptors have been found throughout the reproductive tract (oviduct, myometrium, endometrium, cervix, and the uterine vessels), and their expression is dynamic, changing during the estrous cycle (reviewed by Shemesh, 2001). This author stated that there is a direct temporal relationship between the increased concentration of LH receptors, induction of cyclooxygenase 2, and $PGF_{2\alpha}$ production in endometrial epithelial cells, implying a role for LH in the initiation of bovine luteolysis. In that review it was proposed that LH receptor protein and its mRNA are expressed maximally in the endometrium, myometrium, and cervix in the late luteal phase as well as in lower concentrations in the postovulatory period and minimally during the proestrus–estrus phase. In contrast, the expression pattern of LH receptor protein and its mRNA in the uterine vein moves in the opposite direction. These findings may suggest that the administration of hCG as early as d 2 after FTAI, with high affinity to bind to LH receptors, may increase $PGF_{2\alpha}$ production by the endometrial cells in "acyclic" cows. In a previous study from our group (Beltman et al., 2009), we reported that the administration of $PGF_{2\alpha}$ on d 3, 3.5, and 4 of the estrous cycle induced low production of P4 while maintaining the CL functionality. This may explain why we did not observe differences in P4 concentrations in this group of cows compared with the cows with similar CL status in the control. Therefore, distinct expression pattern of the LH receptors in cows under different physiological conditions, such as ovarian cyclicity resumption (cyclic, acyclic), parity (nulliparous, primiparous, multiparous), lactation, and—probably a key point—the day of the estrus cycle in the moment of hCG administration, could explain the inconsistent reports of the effect of hCG on fertility. This area merits further study.

CONCLUSIONS

The key role of P4 in driving endometrial gene expression and uterine receptivity in ruminants is well established (Lonergan et al., 2016; Spencer et al., 2016). A large number of studies have attempted to

improve pregnancy rate through administration of hCG on d 5 postinsemination to induce ovulation of the first wave dominant follicle and formation of an accessory CL. Here, we targeted an increase in P4 production by the nascent CL through hCG administration on d 2 post-AI. Despite stimulating an increase in circulating P4 concentrations, reproductive performance was not improved by administering hCG to lactating dairy cows. It is apparent from the findings in the present study and from previous publications that the effect of hCG administration varies depending on physiological status of the animal and the exact timing of hCG administration.

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