Administration of prostaglandin F_{2α} 14 d before initiating a G6G or a G7G timed artificial insemination protocol increased circulating progesterone prior to artificial insemination and reduced pregnancy loss in multiparous Holstein cows

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ABSTRACT

The objectives of this study were to determine the effect of PGF_{2α} treatment 14 d before the initiation of a G6G or G7G (PGF_{2α}, 2 d, GnRH, 6 or 7 d, Ovsynch) protocol on ovarian response, synchronization protocol on ovarian response, progesterone (P₄) concentration, pregnancy per AI (P/AI), and pregnancy loss in multiparous Holstein cows. Cows (3.6 ± 1.1 lactations and yielding >30 kg/d of milk) were randomly assigned to 1 of 4 timed artificial insemination (TAI) protocols: 1) G6G (n = 240), one injection of PGF_{2α}, GnRH 2 d later and a 7-d Ovsynch protocol (GnRH, 7 d, PGF_{2α}, 56 h, GnRH 16 h TAI) was initiated 6 d later; 2) PG6G (n = 250), PGF_{2α} 14 d before the initiation of the G6G protocol; 3) G7G (n = 200), one injection of PGF_{2α}, GnRH 2 d later, and a 7-d Ovsynch protocol initiated 7 d later; and (4) PG7G (n = 200), a PGF_{2α} injection 14 d before the initiation of the G7G protocol. Blood samples from a subset of 269 cows were collected at the times of first and second GnRH, and PGF_{2α} of the Ovsynch protocol to measure P₄. Ultrasound examinations were performed to evaluate ovarian response to GnRH and PGF_{2α} of Ovsynch, and to determine pregnancy status at 32 and 60 d after TAI. The proportion of cows with high (≥1 ng/mL) P₄ at first GnRH of Ovsynch was greater for PG6G and PG7G compared with G6G and G7G groups (combined 79.7 vs. 59.3%). In addition, mean (±SEM) plasma P₄ concentration (ng/mL) at PGF_{2α} of Ovsynch was also greater in PG6G (6.5 ± 0.2) and PG7G (6.7 ± 0.3) compared with G6G (5.1 ± 0.2) and G7G (5.0 ± 0.2). Cows given

PGF_{2α} 14 d before initiating a G6G or a G7G TAI (PG6G and PG7G) tended to have a greater P/AI at 32 d compared with those cows not receiving PGF_{2α} (G6G and G7G). However, P/AI at 60 d was greater in cows subjected to PG6G and PG7G protocols (31.1 vs. 39.2%), with a lower pregnancy loss between 32 and 60 d (11.65 vs. 19.7%). In summary, administration of PGF_{2α} 14 d before initiating a G6G or a G7G TAI protocol increased P₄ concentrations before artificial insemination and late embryonic/early fetal survival in multiparous Holstein cows.

Key words: multiparous Holstein cow, G6G, progesterone, ultrasonography

INTRODUCTION

As the expression of estrus is very poor in lactating dairy cows, and overall estrus detection efficiency is low, timed AI (TAI) protocols are implemented to improve dairy herd reproductive performance. Several GnRH-based protocols that facilitate TAI without the necessity to detect estrus have been developed and are currently available for dairy producers (Colazo and Mapletoft, 2014). Most of those protocols involve presynchronization with 2 PGF_{2α} treatments to ensure the presence of a functional dominant follicle at the beginning of the Ovsynch protocol (Presynch/Ovsynch; Moreira et al., 2001; El-Zarkouny et al., 2004). A short presynchronization protocol that consists of a single administration of PGF_{2α} and GnRH 2 d later 6 d before Ovsynch (G6G) optimized synchronization rate and improved fertility to TAI (Bello et al., 2006). In that regard, 84.6% of cows ovulated to first GnRH of Ovsynch, 96.2% responded to PGF_{2α}, and 50.0% became pregnant following a G6G protocol (Bello et al., 2006). An interval of 7 d between the first 2 GnRH treatments (G7G) would be more practical and easier to use by farm personnel because the majority of treat-

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ments are given the same day of the week. However, pregnancy per AI (**P/AI**) between G6G and G7G protocols has not been compared.

Although combining $PGF_{2\alpha}$ and GnRH for presynchronization might be beneficial in acyclic cows, the G6G protocol resulted in similar P/AI compared with Presynch/Ovsynch in grazing dairy cows (Ribeiro et al., 2012). Based on plasma progesterone concentrations, Presynch/Ovsynch was more efficacious than G6G in cyclic cows, but the opposite was observed in acyclic cows (Ribeiro et al., 2012). Recently, a presynchronization protocol that combines 2 $PGF_{2\alpha}$ 14 d apart and a single GnRH treatment given 4 d after second $PGF_{2\alpha}$ decreased the percentage of acyclic cows and increased fertility over the Presynch protocol in heat-stressed multiparous lactating dairy cows (Dirandeh et al., 2015). Collectively, these studies would indicate that a presynchronization protocol with 2 $PGF_{2\alpha}$ 14 d apart is an acceptable approach for cyclic cows, but the incorporation of GnRH might be beneficial for acyclic or multiparous cows (or both) and further optimize the ovarian response to Ovsynch in cyclic cows (Dirandeh, 2014; Dirandeh et al., 2015). Thus, higher rates of ovulatory response and synchronization, and in turn enhanced fertility following TAI would be expected, in G6G- or G7G-treated cows that receive an extra $PGF_{2\alpha}$ treatment. The objective was to determine the effect of a single $PGF_{2\alpha}$ treatment 14 d before the initiation of a G6G or G7G synchronization protocol on ovarian response, progesterone (P_4) concentration, P/AI, and pregnancy loss in multiparous Holstein cows. The first hypothesis was that the inclusion of a $PGF_{2\alpha}$ treatment 14 d before the initiation of a G6G or G7G TAI protocol would increase fertility by increasing plasma progesterone concentrations preceding TAI and improving synchronization rate to the protocols. The second hypothesis was that P/AI would not differ between G6G and G7G protocols.

MATERIALS AND METHODS

Animals and Treatments

This experiment was conducted at a commercial dairy farm in Iran. A total of 890 multiparous lactating Holstein cows (yielding >30 kg of milk/d) without any sign of clinical illnesses (rectal examination of all cows confirmed no clinical abnormalities of the uterus or abnormal vulva discharge) were enrolled in the study. Cows had an average of 3.6 ± 1.1 lactations (mean $\pm$ SEM) in parity and a BCS ranging from 2.5 to 3.5 (1 = emaciated to 5 = obese). Cows were housed in freestall barns with fans and bedded with sand. Cows received a TMR formulated for lactating dairy cows producing 40

kg of 3.5% fat milk according to NRC (2001) guidelines and had free access to water. Diets were fed twice daily (0700 and 1600 h) for ad libitum intake (10% of refusals on as-fed basis). Main ingredients were silage (corn and alfalfa), grain (barley or corn), hay (alfalfa or grass), and mineral supplements. All cows participating in this experiment were milked thrice daily at approximately 8-h intervals, and monitored daily for signs of diseases. If any health issues occurred, animals were moved to hospital pens and appropriate treatments were performed (following standard treatment protocols) and cows removed from the rest of study.

TAI Protocols

Every week, a cohort of cows was randomly assigned to receive 1 of 4 TAI protocols: **G6G** ($n = 240$), **PG6G** ($n = 250$), **G7G** ($n = 200$), or **PG7G** ($n = 200$). Cows in the G6G group received $PGF_{2\alpha}$ (500 μ g of cloprostenol, i.m.; Parnell Technologies Pty. Ltd., Alexandria, Australia) 2 d later GnRH (100 μ g of gonadorelin acetate, i.m.; Parnell Technologies) followed 6 d later by a modified Ovsynch (Ovsynch56; Brusveen et al., 2008) that consisted of GnRH followed by $PGF_{2\alpha}$ 7 d later and a second GnRH treatment administered 56 h after $PGF_{2\alpha}$. Cows in the PG6G group received the same treatments as the previous group with an additional $PGF_{2\alpha}$ treatment 14 d before initiating the G6G protocol. Cows in the G7G and PG7G groups received same treatments as G6G and G7G groups, respectively, except the Ovsynch was initiated 7 d after first GnRH treatment. All cows were TAI approximately 16 h following second GnRH of Ovsynch. Two professional AI technicians performed all inseminations, with semen from 3 commercially available sires equally balanced among the 4 experimental groups. Treatment protocols and activities during this study are shown in Figure 1.

Ultrasonographic Examinations

Ovarian examinations were performed by transrectal ultrasonography (BCF equipped with a 6- to 8-MHz linear transducer; Ultrasound Australas, Victoria, Australia) during the Ovsynch treatments (at first and second GnRH and at $PGF_{2\alpha}$ administration) and 7 d after TAI in a subset of 490 cows to determine ovarian response to treatments. The proportion of cows ovulating to first and second GnRH of Ovsynch was determined as previously described by Colazo et al. (2009) and Dirandeh et al. (2015). Synchronization rate was calculated as the number of cows responding to $PGF_{2\alpha}$ and ovulating after second GnRH of Ovsynch over the total number of cows as previously described by Dirandeh et al. (2015). A cow was considered synchronized if

Figure 1. Diagram of activities and treatments during the study. Lactating Holstein cows ($n = 890$) were randomly assigned to 1 of the 4 timed AI (TAI) protocols. Cows were subjected to either a G6G or G7G TAI protocol, about one-half of them received a PGF_{2α} treatment 14 d before the initiation of a G6G or G7G (PG6G and PG7G, respectively). Transrectal ultrasonography (U/S) was performed in 490 cows during the Ovsynch and 7 d after TAI to determine ovarian response to treatments and at 32 d after TAI to determine pregnancy. Blood samples (shaded circles) from a subset of 269 cows were collected during the Ovsynch to determine plasma progesterone (P₄) concentrations.

she responded to PGF_{2α} [corpus luteum (CL) ≤ 10 mm at second GnRH of Ovsynch] and ovulated after second GnRH of Ovsynch (presence of a CL 7 d after TAI). Pregnancy diagnosis was performed by ultrasonography at 32 d after TAI. Pregnancy was characterized by the presence of fluid, an embryo, and a heartbeat. Cows diagnosed pregnant at 32 d were re-examined by rectal palpation at 60 ± 5 d after TAI to confirm pregnancy. Pregnancy loss was considered to have occurred when a cow was diagnosed pregnant at 32 d after TAI and not pregnant at 60 d.

Blood Collection and Hormone Assay

Blood samples from a subset of cows (G6G = 70, PG6G = 59, G7G = 80, PG7G = 60) were collected by coccygeal venipuncture at the times of the first and

second GnRH, and PGF_{2α} injections of the Ovsynch protocol. Samples collected into evacuated tubes containing EDTA (10.5 mg, Monoject; Sherwood Medical, St. Louis, MO) and immediately centrifuged at $2,600 \times g$ for 30 min at 4°C. Plasma was separated and stored at -20°C until P₄ concentrations were determined by an ELISA kit (Diaplus, North York, Ontario, Canada). Inter- and intraassay coefficients of variation were 2.2 and 3.3%, respectively. Luteal regression was defined to occur when plasma P₄ concentration was ≥ 1 ng/mL immediately before PGF_{2α} treatment and decreased to <1 ng/mL 56 h later (at second GnRH treatment).

Statistical Analyses

All data were analyzed using SAS (version 9.3 for Windows, SAS Institute Inc., Cary, NC). Pregnancy

Table 1. Effect of timed AI (TAI) protocol on mean ($\pm$ SEM) plasma progesterone (P₄) concentration (ng/mL), percentage of cows with P₄ $\geq$ 1 ng/mL at first GnRH, and PGF_{2α} of Ovsynch and percentage of cows with luteal regression at second GnRH

Item	TAI protocol				P-value
	G6G	PG6G	G7G	PG7G	
P ₄ concentration, ng/mL ($\pm$ SEM)					
at first GnRH	2.2 $\pm$ 0.07 ^a	3.9 $\pm$ 0.15 ^b	2.3 $\pm$ 0.07 ^a	4.1 $\pm$ 0.20 ^b	0.03
at PGF _{2α}	5.1 $\pm$ 0.24 ^a	6.5 $\pm$ 0.22 ^b	5.0 $\pm$ 0.23 ^a	6.7 $\pm$ 0.31 ^b	0.03
at second GnRH	0.6 $\pm$ 0.07 ^a	0.2 $\pm$ 0.01 ^b	0.5 $\pm$ 0.07 ^a	0.1 $\pm$ 0.05 ^b	0.01
Cows with P ₄ $\geq$ 1 ng/mL, % (no.)					
at first GnRH	60.0 (42/70) ^a	77.9 (46/59) ^b	58.7 (47/80) ^a	81.6 (49/60) ^b	0.01
at PGF _{2α}	70.1 (47/67) ^a	84.2 (48/57) ^b	68.7 (55/80) ^a	85.0 (51/60) ^b	0.04
Luteal regression after PGF _{2α} of Ovsynch, ¹ % (no.)	65.6 (44/67) ^a	77.1 (44/57) ^b	63.7 (51/80) ^a	78.3 (47/60) ^b	0.04

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹Luteal regression was defined to occur when plasma P₄ concentration was $\geq$ 1 ng/mL immediately before PGF_{2α} treatment and decreased to $<$ 1 ng/mL 56 h later (at second GnRH treatment).

status at 32 and 60 d after TAI and pregnancy loss between 32 and 60 d were analyzed using a general estimating equations method of the GENMOD procedure. Model specifications included a binomial distribution, logit link function, repeated statement with subject equal to cow, and an exchangeable correlation structure. Administration of PGF_{2α} 14 d before initiation of TAI protocol (yes vs. no), length of the interval between the GnRH of presynchronization and the initiation of Ovsynch (6 vs. 7 d), and their interaction were included in the model. Model diagnostics included visual examination of the raw and standardized residuals. The residuals were plotted against predicted values of each observation. Rankit plots and Wilk-Shapiro tests were used to assess the normality of the residuals. The ratio of the final-model deviance to the model degrees of freedom was also examined.

Ovarian response to treatments during the Ovsynch (response to first and second GnRH, and PGF_{2α}; all based on ultrasonographic examinations) and synchronization rate were analyzed in a subset of 490 cows by GENMOD procedure as well. The model also included administration of PGF_{2α} 14 d before initiation of TAI protocol (yes vs. no), length of the interval between the GnRH of presynchronization and the initiation of Ovsynch (6 vs. 7 d), and their interaction.

Luteal regression (based on plasma P₄) and percentage of cows with P₄ $\geq$ 1 ng/mL at first GnRH and PGF_{2α} treatment of Ovsynch were analyzed in a subset of 269 cows using the LOGISTIC procedure. Plasma P₄ concentrations were analyzed using the MIXED procedure. To detect 10 or 12 percentile differences in pregnancy rate (95% confidence; 80% power), sample sizes of 260 or 196 cows per treatment, respectively, were required (Win Episcopo 2.0, 2001). Probability values $\leq$ 0.05 were considered significant, and those between 0.051 and 0.1 were considered trends.

RESULTS

The effect of TAI protocol on plasma P₄ concentrations is shown in Table 1. The proportion of cows with high plasma P₄ ($>$ 1 ng/mL) was greater for PG6G (77.9 and 84.2) and PG7G (81.6 and 85.0) compared with G6G (60.0 and 70.1) and G7G (58.7 and 68.7) at first GnRH ($P = 0.01$) and PGF_{2α} of Ovsynch (respectively, $P = 0.04$; Table 1). Luteal regression after PGF_{2α} treatment was affected ($P = 0.04$) 77.1% by TAI protocol in cows subjected to PG6G and PG7G vs. 64.6% in cows subjected to G6G and G7G (Table 1). However, overall plasma P₄ concentrations at second GnRH of Ovsynch were lower ($P = 0.01$) in cows given PGF_{2α} 14 d before the initiation of G6G or G7G (Table 1).

Ovulatory response to first GnRH of Ovsynch was greater ($P = 0.01$) in cows in PG6G and PG7G groups compared with G6G and G7G groups (Table 2). Ovulatory response to second GnRH of Ovsynch ($P = 0.07$) and overall synchronization rate ($P = 0.08$) tended to be lower in cows in PG6G and G6G protocols compared with those cows in PG7G and G7G (Table 2).

Cows given PGF_{2α} 14 d before initiating a G6G or a G7G TAI (PG6G and PG7G) tended ($P = 0.09$) to have a greater P/AI at 32 d compared with those cows no receiving PGF_{2α} (G6G and G7G; Table 3). However, P/AI at 60 d was greater ($P = 0.01$) in cows subjected to PG6G and PG7G protocols because lower pregnancy loss was lower between 32 and 60 d in those groups ($P = 0.03$; Table 3).

DISCUSSION

This study was designed to determine the effect of a single PGF_{2α} treatment 14 d before the initiation of a G6G or a G7G synchronization protocol on ovarian response, plasma P₄ concentration, P/AI, and pregnancy

Table 2. Number and percentage of lactating Holstein cows that ovulated after first and second GnRH of Ovsynch, and were considered synchronized in the 4 TAI protocols

Item	TAI protocol				<i>P</i> -value ¹		
	G6G	PG6G	G7G	PG7G	PGF _{2α}	Length	Interaction
Total no. cows	140	150	100	100			
Cows that ovulated to first GnRH of Ovsynch, ² % (no.)	69.2 (97)	80.6 (121)	68.0 (68)	81.0 (81)	0.01	0.58	0.75
Cows that ovulated to second GnRH of Ovsynch, ³ % (no.)	71.4 (100)	80.0 (120)	69.0 (69)	81.0 (81)	0.35	0.07	0.42
Synchronization rate, ⁴ % (no.)	67.1 (94)	75.3 (113)	69.0 (69)	78.0 (78)	0.30	0.08	0.19

¹The main effects were administration of PGF_{2α} 14 d before initiation of TAI protocol and length of the interval between the GnRH of presynchronization and the initiation of Ovsynch (6 vs. 7 d).

²Ovulation was determined retrospectively by recording the location, number, and size of follicles and CL at first GnRH, and comparing those data to ovarian structures on the day of PGF_{2α} of Ovsynch.

³Ovulation was confirmed by the presence of a CL 7 d after TAI in the same ovary, which had the largest follicle at the second GnRH of Ovsynch. Ovulatory response to the second GnRH of Ovsynch tended to be lower in cows in PG6G and G6G protocols compared with those cows in PG7G and G7G.

⁴A cow was considered synchronized if she responded to PGF_{2α} (CL ≤10 mm at second GnRH of Ovsynch) and ovulated after second GnRH of Ovsynch (presence of a CL 7 d after TAI). Synchronization rate tended to be lower in cows in PG6G and G6G protocols compared with those in PG7G and G7G.

loss in multiparous Holstein cows. Cows receiving an injection of PGF_{2α} 14 d before the initiation of a G6G or a G7G had an increased ovulatory response to first GnRH of Ovsynch compared with cows subjected to G6G and G7G protocols. Ovulation to the first GnRH would result in the initiation of a new follicular wave 36 h later (Martínez et al., 1999; Dirandeh et al., 2009) ensuing synchronization of dominant follicle growth and development and improving pregnancy outcome to Ovsynch (Vasconcelos et al., 1999; Bello et al., 2006). It has been reported that day of the estrous cycle (Martínez et al., 1999; Vasconcelos et al., 1999; Moreira et al., 2000), circulating P₄ concentrations (Colazo et al., 2008), and the size of the dominant follicle at the time of GnRH administration (Xu et al., 1995) are important factors that would determine whether ovulation occurs in response to first GnRH treatment. In addition, treatment with PGF_{2α} in the early stages of the

estrous cycle (first 5 d) was found to be ineffective in causing a luteolytic response in cattle (Momont and Seguin, 1984). Consequently, two PGF_{2α} given 14 d apart ensures that cows would have a responsive CL when they received the second PGF_{2α} treatment and a reduced interval to onset of estrus (Rosenberg et al., 1990). Therefore, it is plausible to assume that cows in the PG6G and PG7G were more synchronous with the majority of them likely to be on early diestrus at first GnRH of Ovsynch, when GnRH treatment would be more effective in inducing ovulation (Moreira et al., 2000).

A greater percentage of cows in the PG6G and PG7G groups had plasma P₄ ≥1 ng/mL at first GnRH of Ovsynch. Results from other studies have indicated that a presynchronization protocol that includes GnRH reduces the proportion of animals without a CL at onset of Ovsynch. Stevenson et al. (2012) reported that

Table 3. Effects of timed AI (TAI) protocols on pregnancy per AI (P/AI) at d 32 and 60 after AI and pregnancy loss in multiparous Holstein cows

Item	TAI protocol				<i>P</i> -value ¹		
	G6G	PG6G	G7G	PG7G	PGF _{2α}	Length	Interaction
Total no. of cows	250	250	200	200			
P/AI at 32 d after AI, % (no.)	39.5 (95)	40.4 (101)	38.0 (76)	48.5 (97)	0.09	0.34	0.14
P/AI at 60 d after AI, % (no.)	31.2 (75) ^a	36.4 (89) ^b	31.0 (62) ^a	42.0 (84) ^b	0.01	0.43	0.38
Pregnancy loss after AI, ² % (no.)	21.0 (20/95) ^a	9.9 (10/101) ^b	18.4 (14/76) ^a	13.4 (13/97) ^b	0.03	0.76	0.38

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹The main effects were administration of PGF_{2α} 14 d before initiation of TAI protocol and length of the interval between the GnRH of presynchronization and the initiation of Ovsynch (6 vs. 7 d).

²Pregnancy loss between 32 and 60 d after AI, % (no. not pregnant at 60 d/no. pregnant at 32 d).

a greater percentage of cows subjected to a presynchronization protocol which included a GnRH injection preceded 3 d earlier by PGF_{2 α} treatment (similar to the G7G protocol used here) had $P_4 \geq 1$ ng/mL at first GnRH compared with those subjected to a presynchronization with only PGF_{2 α} . Similarly, 93% of the cows given PGF_{2 α} and GnRH 3 d later had a functional CL at first GnRH of Ovsynch compared with 56% of cows that were at random stages of the estrous cycle at initiation of Ovsynch (Peters and Pursley, 2002). Also, Double-Ovsynch (a protocol that involves 2 Ovsynch protocols following one after the other with the third GnRH treatment being administered 7 d after the second) resulted in fewer cows with $P_4 < 1$ ng/mL compared with the standard Presynch protocol that includes 2 PGF_{2 α} (9.4 vs. 33.3%; Souza et al., 2008). Based on our findings, it is likely that administration of PGF_{2 α} 14 d before the initiation of a G6G or G7G resulted in a significant percentage of these cows with a more functional CL at initiation of Ovsynch. Furthermore, cows subjected to PG6G and PG7G had greater plasma P_4 concentrations at PGF_{2 α} of Ovsynch compared with those subjected to G6G and G7G, suggesting that circulating P_4 was enhanced during follicular development before TAI in PG6G and PG7G cows. Some indications were found that increasing P_4 before AI can result in a substantial improvement in fertility in lactating dairy cows (Folman et al., 1990; Cunha et al., 2008; Colazo et al., 2013), as one of the reasons for lower fertility in those animals is reduced P_4 concentrations (Inskeep, 2004). Although increased plasma P_4 concentrations before TAI were expected to improve fertility of dairy cows, P/AI at 32 d was not affected by TAI protocol. It is not completely clear why P/AI at 32 d was not improved in cows given PGF_{2 α} 14 d before the initiation of a G6G or G7G, but it is possible that the concentration of P_4 in plasma was already high enough in cows not receiving PGF_{2 α} or the difference in plasma P_4 among TAI protocols was not substantial enough to result in a significant improvement in fertility. In this regard, Folman et al. (1990) reported improvements in fertility in lactating dairy cows with plasma P_4 concentrations prior AI above 5.1 ng/mL. Moreover, fertility was improved in lactating cows with plasma concentrations of 4.4 ng/mL of P_4 at PGF_{2 α} of Ovsynch compared with those cows with concentrations of 2.5 ng/mL of P_4 (Cunha et al., 2008). The other possibility is that the statistical power was insufficient to detect statistical significance when the difference in P/AI of cows given PGF_{2 α} (PG6G and PG7G) or not (G6G and G7G) was 5.2 percentage units.

Cows subjected to G6G and G7G had lower P/AI at 60 d, and hence, tended to have greater pregnancy losses between 30 and 60 d after TAI compared with those

subjected to PG6G and PG7G protocols. As discussed previously, cows subjected to G6G and G7G protocols had reduced plasma P_4 concentrations during Ovsynch. Likewise, an increase in pregnancy loss between 29 and 57 d after AI was observed in cows with low P_4 (14.3% loss) before breeding as compared with cows with high P_4 (6.8% loss; Cunha et al., 2008). In addition, circulating P_4 concentrations after AI did not differ between cows that had low P_4 before AI (2.9 ng/mL) and those cows with high P_4 before AI (2.5 ng/mL), indicating that the difference in pregnancy loss was not due to the concentration of P_4 after AI (Cunha et al. 2008). Taken together, the findings from this study and those from Cunha et al. (2008) draw attention to the importance of high P_4 before AI for maintenance of pregnancy.

The overall P_4 concentration at second GnRH of Ovsynch was lower in cows in the PG6G and PG7G groups. It has been demonstrated that elevated concentrations of P_4 near AI, due to inadequate CL regression, will result in a reduction in fertility in cattle. In this regard, Ghanem et al. (2006), found a significant decrease in fertility as well as greater embryonic losses in lactating cows with high milk P_4 at the time of AI. Elevated concentrations of circulating P_4 at the time of AI may affect semen transport and fertilization by reducing uterine contractility given that P_4 decreases the number of oxytocin, angiotensin II, and estrogen receptors in the uterus and antagonizes estrogen induction of estrogen receptors in the myometrium (Graham and Clarke, 1997). Delayed (prolonged P_4 clearance) or incomplete CL regression may also have an indirect negative effect on estradiol-17 β production and pulses of LH (Bridges and Fortune, 2003) that may impair the normal ovulation process. Incomplete luteolysis following PGF_{2 α} treatment has been observed in 10 to 25% of cows treated with GnRH-based protocols (Martins et al., 2011; Dadarwal et al., 2013). In these studies, cows that had very small elevations in circulating P_4 near AI had greatly reduced fertility. In the past, a cutoff of 1 ng/mL has been used to define complete luteolysis. However, it has been recently shown that the probability of pregnancy would be diminished when serum concentrations of P_4 do not decline to <0.5 ng/mL at 48 h following PGF_{2 α} administration (Souza et al., 2007; Brusveen et al., 2009). Hence, a very low circulating P_4 near AI is likely to result not only in increased P/AI but also in improved late embryonic/early fetal survival in lactating dairy cows subjected to GnRH-based protocols.

A greater percentage of cows ovulated to the first GnRH of Ovsynch when treatment was given on d 6 (94%) compared with d 7 (82%) or 8 (73%) after the GnRH treatment of G6G (Wiltbank and Pursley, 2014). This observation would suggest that an interval of 6 d between the first 2 GnRH treatments in a G6G

is more likely to result in higher fertility than intervals of either 7 (G7G) or 8 (G8G) days. However, a difference in P/AI between G6G and G7G protocols has not been previously reported. Bello et al. (2006) reported that 84.6% of cows ovulated to first GnRH of Ovsynch, 96.2% responded to PGF_{2α}, and 50.0% became pregnant following a G6G protocol. In the present study, ovarian responses and P/AI with the G6G protocol was approximately 10 percentile points below of those reported by Bello et al. (2006) but G6G and G7G protocols did not differ. Therefore, based on the results from the present study, we suggest that the G7G protocol be used with confidence and that it would be more practical to implement because the majority of treatments are given the same day of the week.

CONCLUSIONS

In the present study, the percentage of cows that ovulated following the first GnRH treatment of Ovsynch increased when cows were given PGF_{2α} 14 d before initiating a G6G or G7G TAI protocol. In addition to a higher ovulatory response to first GnRH of Ovsynch, administration of PGF_{2α} also increased circulating P₄ concentrations during follicular development before AI and resulted in decreased P₄ near to AI. Despite a significant better ovarian response to treatments and improved circulating P₄ in cow given PGF_{2α}, P/AI at 32 d merely tended to differ among TAI protocols. However, administration of PGF_{2α} 14 d before initiating a G6G or G7G improved P/AI at 60 d by apparently enhancing late embryonic/early fetal survival in multiparous Holstein cows.

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