Effect of a second treatment with prostaglandin $F_{2\alpha}$ during the Ovsynch protocol on luteolysis and pregnancy in dairy cows

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ABSTRACT

The main objective of this study was to evaluate the effect of a second treatment with prostaglandin $F_{2\alpha}$ (PGF) during Ovsynch on regression of the corpus luteum (CL) and on fertility to the timed artificial insemination. Two experiments were performed. In both experiments, cows were randomized to receive (1) no additional treatments with PGF = 1 PGF, or (2) a second PGF treatment at 24 h after the first PGF treatment = 2 PGF. The first experiment ($n = 344$ synchronized lactating dairy cows that received artificial insemination at 81 ± 3 d in milk) used the Double-Ovsynch protocol for synchronizing ovulation. Blood samples were collected at the PGF and final GnRH treatments (72 and 16 h before timed artificial insemination) during the breeding Ovsynch protocol, to determine CL regression in response to the protocol. Treatment with 2 PGF increased CL regression from 83.0% with 1 PGF to 97.0% with 2 PGF. The effect of 2 PGF on CL regression was observed in both primiparous and multiparous cows. Cows with lower (2.0 to 4.8 ng/mL) versus greater (4.9 to 12.0 ng/mL) circulating progesterone at the time of PGF had lower percentage of cows with complete CL regression after 1 PGF (66.7 vs. 88.1%) but not after 2 PGF (95.1 vs. 97.6%). Experiment 2 used 2,148 lactating dairy cows on 11 dairy farms in 4 different regions of the United States. Cows were synchronized with Ovsynch and received timed artificial insemination at 60 ± 3 d in milk. Cows that received 2 PGF had a tendency for increased pregnancies per artificial insemination (P/AI) compared with cows with 1 PGF (36.1 vs. 33.3%). This tendency for improvement in P/AI was observed in multiparous but not in primiparous cows. Combining data from the 2 experiments indicated a 9.45% relative increase in P/AI for cows receiving 2 compared with 1 PGF (37.6 vs.

34.4%) with the increase in P/AI observed in multiparous but not in primiparous cows. Thus, a second PGF treatment in Ovsynch-type protocols can increase pregnancy success by about 10%, primarily due to enhanced fertility in multiparous cows.

Key words: Ovsynch, prostaglandin $F_{2\alpha}$, fertility, reproduction

INTRODUCTION

Reproductive efficiency in lactating dairy cows is suboptimal, due to inadequate service risk and reduced pregnancies per AI (P/AI; Thatcher et al., 2006; Wiltbank et al., 2006; Norman et al., 2009). Timed AI programs have been developed to increase the service risk; however, fertility is still suboptimal when uncomplicated protocols, such as Ovsynch, are used (Pursley et al., 1995, 1997; Rabiee et al., 2005). To practically increase the percentage of cows at an optimal stage of the cycle at the initiation of Ovsynch, presynchronization protocols have been developed such as Presynch-Ovsynch (Moreira et al., 2001; El-Zarkouny et al., 2004; Navanukraw et al., 2004), Double-Ovsynch (Souza et al., 2008; Herlihy et al., 2012; Ayres et al., 2013), and G-6-G (Peters and Pursley, 2002; Bello et al., 2006). Presynchronization protocols that result in improved fertility during timed AI protocols generally result in increased ovulation to the first GnRH of Ovsynch (Galvao et al., 2007; Giordano et al., 2013; Wiltbank and Pursley, 2014). The newly formed corpus luteum (CL) can increase circulating progesterone (P4) during the preovulatory follicle growth phase, potentially increasing fertility and reducing double ovulation rate (Bisinotto et al., 2010; Giordano et al., 2010; Wiltbank et al., 2014b). Indeed, cows that ovulate to the first GnRH treatment generally have greater fertility than cows that do not ovulate (Bello et al., 2006; Chebel et al., 2006; Keskin et al., 2010). However, the new CL, induced by the first GnRH treatment of Ovsynch, may be difficult to regress with a single treatment with prostaglandin $F_{2\alpha}$ (PGF). Lack of complete regression

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of the CL to the PGF treatment has been observed in 10 to 25% of cows treated with Ovsynch (Brusveen et al., 2009; Martins et al., 2011; Wiltbank and Pursley, 2014). In these studies, cows that have small elevations in circulating P4 near AI, due to inadequate CL regression, have reduced fertility. This is particularly important if a shortened protocol is used, such as an Ovsynch or Cosynch protocol with only 5 d between the GnRH and PGF treatment (5-d protocol) instead of the normal 7-d interval (Santos et al., 2010; Stevenson et al., 2013, 2014), because a younger CL could be present at the time of PGF treatment and the young CL is difficult to regress with a single PGF treatment (Santos et al., 2010; Ribeiro et al., 2012; Nascimento et al., 2014). However, even in the 7-d Ovsynch protocol, lack of regression of the CL is an important problem, leading to reductions in fertility during Ovsynch or modifications of Ovsynch protocols (Martins et al., 2011; Giordano et al., 2012b; Wiltbank et al., 2014b). As would be expected, incomplete CL regression is more likely in cows with a new CL than in cows that did not ovulate to the first GnRH treatment (Giordano et al., 2012b).

To overcome the problem with inadequate CL regression during timed AI protocols, 2 general strategies have been used. One strategy is to give an increased dose of PGF during either the 5- or 7-d Ovsynch protocols (Ribeiro et al., 2012; Giordano et al., 2013). A second strategy is to give a second dose of PGF on the subsequent day after the first PGF treatment of a 5- or 7-d Ovsynch protocol. In the 5-d Ovsynch protocol, fertility was reduced if only a single PGF was given (Santos et al., 2010) or if 2 PGF treatments were given on the same d (5 d after GnRH) compared with giving one treatment on d 5 and a second on d 6 (Ribeiro et al., 2012). However, in the 7-d protocol, increasing the dose of cloprostenol from 500 to 750 μ g increased CL regression in multiparous but not primiparous cows and tended to increase fertility (Giordano et al., 2013). Another study using the 7-d Ovsynch protocol, compared CL regression and fertility in cows treated with 1 PGF (d 7) or 2 PGF treatments (d 7 and 8; Brusveen et al., 2009). An increased percentage of cows with complete CL regression (<0.4 ng/mL 56 h after PGF) was found after 2 (326/341 = 95.6%) compared with 1 (301/356 = 84.6%) PGF treatment. However, no improvement in fertility was detectable, statistically. This study was probably statistically underpowered ($n = 772$) because the 5.7% improvement in fertility that was observed in first service cows (52.7 vs. 47.0%) was of the expected magnitude but was not statistically significant (Brusveen et al., 2009). Thus, evaluation of the effect of a second dose of PGF on fertility needs to be further evaluated using a larger sample size.

The present study was designed to test the hypothesis that fertility will be improved in cows treated with 2 doses of PGF, one on d 7 and a second on d 8 (**2 PGF**), compared with a single dose of PGF on d 7 (**1 PGF**) of the Ovsynch protocol. Two experiments were performed. The first experiment was done on a single dairy farm in cows that were synchronized with the Double-Ovsynch protocol and appropriate blood samples were collected to determine if 2 PGF resulted in greater CL regression. The second experiment used the Ovsynch protocol, without any presynchronization, in a large number of commercial dairy farms to evaluate whether treatment with 2 PGF could improve fertility to the protocol.

MATERIALS AND METHODS

All procedures were approved by the Animal Care Committee of the College of Agriculture and Life Sciences, University of Wisconsin–Madison.

Experiment 1

A total of 373 lactating Holstein cows (172 primiparous; 201 multiparous) were synchronized with Double-Ovsynch as previously described (Souza et al., 2008; Herlihy et al., 2012; Ayres et al., 2013). All cows were synchronized using a PGF analog (Cloprostenol sodium; 250 μ g/mL; Estroplan) and GnRH (gonadorelin acetate; 100 μ g/mL; Gonabreed) provided by Parnell (Overland Park, KS). Briefly, cows were treated with Double-Ovsynch starting at 51 to 57 DIM to receive fixed-time AI (**FTAI**) at 78 to 84 DIM (GnRH–7 d–PGF–3 d–GnRH–7 d–GnRH–7 d–PGF–56 h–GnRH–16 h–FTAI). At the time of the final PGF, cows were randomized to 1 of 2 treatment groups: 1 PGF = no additional PGF treatments; 2 PGF = second PGF treatment 24 h after first PGF of breeding Ovsynch. Pregnancy diagnosis was done by transrectal ultrasonography at 32 d after FTAI. Experimental protocol is shown in Figure 1.

Blood samples were taken at the time of the first PGF treatment of the breeding Ovsynch protocol (72 h before FTAI) and at the time of the final GnRH treatment (16 h before FTAI). Blood samples were collected from the coccygeal vessels into vacuum tubes. Following collection, the blood samples were placed immediately on ice and transported on ice to the laboratory. After clotting (~ 6 h), samples were centrifuged at $1,900 \times g$ for 20 min at 4°C and serum was isolated into vials, frozen, and stored at -20°C until assayed for P4 concentrations. Progesterone was determined directly from serum using a solid phase RIA kit (Coat-A-Count,

Siemens Healthcare Diagnostics, Los Angeles, CA) with no extraction. The assay had a sensitivity of 0.04 ng/mL and a coefficient of variation of 2.2%. Results are expressed in nanograms per milliliter.

Experiment 2

This experiment was performed on 11 commercial dairies in different locations in the United States: 3 in Wisconsin, 3 in California, 2 in New York, 1 in Pennsylvania, 1 in New Mexico, and 1 in Texas. Although overall management was quite different for each herd, each herd used the same reproductive management protocol during this experiment. Cows were randomly assigned to 1 of 3 treatment groups within the weekly cohort of a herd using only parity as a blocking factor. One of the treatment groups, Presynch-Ovsynch with heat detection, was not considered in the present analysis but will be presented in a subsequent manuscript. Therefore, only 2 treatment groups were used for all analyses, as shown in Figure 1. One treatment group received Ovsynch with GnRH (gonadorelin acetate; 100 µg/mL; Gonabred, Parnell) treatment at 50 ± 3 DIM, followed 7 d later by PGF treatment (cloprostenol sodium; 250 µg/mL; Estroplan; Parnell), followed ~56 h later by a second treatment with GnRH, and a FTAI at ~16 h after the GnRH treatment (1 PGF). The second treatment group (2 PGF) also received the same Ovsynch treatments but in addition received a second PGF 24 h after the first PGF treatment. Pregnancy diagnosis was performed with ultrasound utilizing the normal schedule for each herd (WI and PA herds: 31–33 d after AI; CA, NY, NM, TX herds: 37–43 d after AI). Data on all treatments, AI, and pregnancy diagnosis were recorded

for each individual cow in most cases ($n = 1,912$). In some cows, most data were recorded accurately, but 1 ($n = 110$) or 2 or more treatments ($n = 126$) were not recorded in a cow. Data from these cows were analyzed separately, found to be similar to the rest of the cows, and therefore all of these cows were used in the final analyses. Pregnancy was based on a verified pregnancy diagnosis. Nonpregnancy was based on absence of pregnancy at a pregnancy diagnosis or a rebreeding to an estrus before pregnancy diagnosis.

Statistical Analyses

Statistical analyses were performed with SAS (version 9.4, SAS Institute Inc., Cary, NC). Variables with a binomial distribution, such as P/AI and percentage of cows with CL regression, were analyzed by logistic regression using the LOGISTIC procedure. Continuous variables were analyzed using ANOVA. Logistic regression was used to compare the relationship between circulating P4 and the binomial traits. In experiment 1, cows with <2.0 ng/mL of P4 on day of PGF were considered as not having been synchronized by the Double-Ovsynch protocol ($n = 29$; 13 primiparous, 16 multiparous). Analyses in Tables 2 and 6 were done with all cows treated with Double-Ovsynch, whereas those in Tables 1 and 3 were done only with synchronized cows (total of 344 cows; $n = 159$ primiparous; $n = 185$ multiparous). Treatment and parity were forced into the statistical models and factors were kept in the model if $P < 0.15$. No significant effects were found of farm and farm by treatment interaction, and therefore these interactions were not kept in the final statistical analysis, although farm remained in the model.

Figure 1. Schematic diagram of the experimental protocols used for experiments (EXPT.) 1 and 2. In EXPT. 1, cows began Double-Ovsynch at 54 ± 3 DIM with timed AI at 81 ± 3 DIM. In EXPT. 2, cows began Ovsynch at 50 ± 3 DIM with timed AI at 60 ± 3 DIM. PGF = prostaglandin $F_{2\alpha}$.

Our hypothesis was that an additional PGF would increase percentage of cows with CL regression and percentage of cows pregnant to the FTAI, based on previous results. Therefore, 1-tailed comparisons were used throughout the study for comparison of effect of 2 PGF on these variables. Previous studies observed parity effects on response to PGF (Martins et al., 2011; Giordano et al., 2013). Therefore, results were analyzed for all combinations of parities and treatment. Statistical differences were considered significant at $P \leq 0.05$ and as a tendency for $P \leq 0.10$.

RESULTS

Experiment 1

Circulating P4 concentrations on the day of PGF were similar for cows receiving 1 PGF (7.0 ± 0.2 ; $n = 176$) or 2 PGF (7.1 ± 0.2 ; $n = 168$). Overall, primiparous had greater circulating P4 concentrations than multiparous (7.7 ± 0.2 vs. 6.5 ± 0.2 ; $P = 0.0001$) cows. Treatment with either 1 or 2 PGF resulted in reduced circulating P4 concentrations in all cows in both treatments. The circulating P4 concentrations at 56 h after PGF (day of final GnRH) were greater ($P = 0.005$) for cows that received 1 PGF (0.4 ± 0.04) compared with 2 PGF (0.2 ± 0.05).

The percentage of cows with complete CL regression (<0.5 ng/mL at 56 h after PGF; Wiltbank et al., 2014b) is shown in Table 1. Overall, no differences were found between parities in the percentage of cows with complete CL regression. More cows were found with CL regression following 2 compared with 1 PGF. This treatment effect on CL regression was observed when all cows were used in the analysis ($P = 0.0001$) or when only primiparous ($P = 0.001$) or multiparous ($P = 0.006$) cows were used in the analysis.

The distribution of cows based on circulating P4 at the time of the final GnRH treatment is shown in Figure 2. Overall, a greater ($P < 0.0001$) percentage of cows was found with higher P4 (≥ 0.5 ng/mL) in

Figure 2. Comparison of effect of 1 versus 2 prostaglandin $F_{2\alpha}$ (PGF) treatments on the distribution of cows by circulating progesterone (P4) at the time of final GnRH treatment using data from cows that were synchronized, as shown by elevated P4 at time of PGF (≥ 2 ng/mL), in experiment 1 ($n = 344$).

1 PGF (17.0%; 30/176) than 2 PGF (2.4%; 4/168). Conversely, a greater ($P = 0.006$) percentage of cows was observed with lower P4 (0.1 to 0.19) for 2 PGF (49.4%; 83/168) than 1 PGF (34.7%; 61/176). If the comparison used all cows below 0.2 ng/mL, a greater ($P = 0.006$) percentage of cows was also found with lower P4 following treatment with 2 (60.7%; 102/168) compared with 1 (42.0%; 74/176) PGF.

Overall, no statistically significant treatment differences were found in P/AI (Table 2). In addition, no statistical differences were found in P/AI between parities for 1 PGF, 2 PGF, or for all cows.

Figure 3 shows the relationships between circulating P4 concentrations at the time of first PGF and the probability of CL regression and P/AI in 1 PGF compared with 2 PGF. For CL regression (Figure 3A), cows with lower P4 at the time of first PGF treatment had a reduced percentage of cows with complete CL regression for 1 PGF ($P = 0.02$). In contrast, for 2 PGF, no effect ($P = 0.44$) was found of circulating P4 at the time of PGF on percentage of cows with CL regression with almost all cows having complete CL

Table 1. Effect of 1 versus 2 treatments with prostaglandin $F_{2\alpha}$ (PGF) on percentage of cows with complete regression of the corpus luteum in primiparous and multiparous cows synchronized with Double-Ovsynch (experiment 1)

% of cows with complete CL regression ¹ (no./no.)	1 PGF	2 PGF	P-value
Primiparous	81.2 (65/80)	97.5 (77/79)	0.001
Multiparous	84.4 (81/96)	96.7 (86/89)	0.006
P-value	0.69	1.0	
Overall	83.0 (146/176)	97.0 (163/168)	0.0001

¹Circulating progesterone <0.5 ng/mL at 56 h after first PGF in cows that had ≥ 2.0 ng/mL of progesterone on the day of first PGF.

regression, regardless of circulating P4 at time of first PGF treatment.

Figure 3B shows that both groups of cows had the lowest P/AI in cows that had lower P4 at the first PGF treatment. For 1 PGF, no effect ($P = 0.13$) was found of increasing P4 at the time of PGF on increasing P/AI. For 2 PGF, greater ($P = 0.02$) P/AI was found with increasing circulating P4 at the time of PGF treatment. In addition, when all cows (1 or 2 PGF) are combined, P/AI increases with increasing circulating P4 at the time of initial PGF treatment (data not shown; $P = 0.01$). Figure 3C also shows the P/AI but including only cows that had complete luteolysis at the time of final GnRH treatment. An effect was found of P4 at the time the first PGF on P/AI in 2 PGF cows ($P = 0.03$) but not in 1 PGF cows ($P = 0.49$).

The relationship of P4 at the first PGF treatment with percentage of cows with complete CL regression could be further illustrated by doing a quartile analysis, based on circulating P4 at the time of first PGF treatment (Table 3). For 1 PGF, cows in quartile 1 (lowest P4 concentrations; 2.0 to 4.8 ng/mL) had lower ($P = 0.002$) CL regression (66.7%; 28/42) compared with the other 3 quartiles (88.1%; 118/134), which did not differ from each other. In contrast for 2 PGF, quartile 1 (95.1%; 39/41) had similar ($P = 0.60$) CL regression as observed for the other 3 quartiles receiving 2 PGF (97.6%; 124/127). Further, a comparison between cows receiving 1 versus 2 PGF within quartiles demonstrated that 1 PGF had lower CL regression than 2 PGF, for quartile 1 (66.7 vs. 95.1%; $P = 0.002$) or for the combination of quartiles 2, 3, and 4 (88.1 vs. 97.6%; $P = 0.0034$).

Quartile analysis also demonstrated that cows with lower P4 at the time of PGF (quartile 1) had similar P/AI for cows receiving 1 (31.0%; 13/42) compared with 2 (31.7%; 13/41) PGF (Table 3). In contrast, for quartiles 2, 3, and 4, a tendency ($P = 0.10$) was observed for decreased P/AI in cows receiving 1 (44.0%; 59/134) compared with 2 (52.8%; 67/127) PGF. This numerical difference between 1 and 2 PGF could be observed in quartile 2 (40.0 vs. 46.7%; $P = 0.34$), quartile 3 (47.7

vs. 55.8%; $P = 0.30$), and quartile 4 (44.4 vs. 56.4%; $P = 0.19$).

Experiment 2

The results from all 11 farms are shown in Table 4. Each of the farms had at least 100 cows assigned to the experiment. One of the farms (NY-06) had a significant positive effect of treatment on results, whereas other farms had no significant effect of treatments within the farm. Overall, by simple chi-square analysis, a tendency ($P = 0.068$) was found for an effect of treatment on P/AI with an increase from 33.3% in 1 PGF compared with 36.1% in 2 PGF.

In the overall logistic regression model, no significant effect was observed of farm on the results ($P = 0.71$). Also, no interaction was found of parity by treatment ($P = 0.74$) or farm by treatment ($P = 0.68$) in the overall model. Farm was kept in the final logistical model; however, the interactions were not used in the final model. In the final logistic model, a significant effect of parity ($P = 0.03$) was found and a tendency was observed for an effect of treatment (0.078). The results, separated by parity, are shown in Table 5. Treatment had no effect in primiparous cows ($P = 0.39$). However, a tendency was observed for an effect of treatment in multiparous cows ($P = 0.073$). When multiparous cows were evaluated by parity, an effect was found of treatment in cows of second and third lactation ($P = 0.047$) but no effect of treatment in older cows (fourth or greater lactations; Table 5).

Combined Results of Experiments 1 and 2

The 2 experiments were done with a similar experimental design, and therefore results were combined to more thoroughly test the effect of a second PGF treatment in the Ovsynch protocol on P/AI (Table 6). No effect was observed of 2 PGF compared with 1 PGF on P/AI in primiparous cows ($P = 0.39$). However, in multiparous cows, 2 PGF had increased ($P = 0.043$) P/AI compared with 1 PGF by 12.31%. This effect of a

Table 2. Effect of 1 versus 2 treatments with prostaglandin $F_{2\alpha}$ (PGF) on percentage pregnant/AI (P/AI) in primiparous and multiparous cows synchronized with Double-Ovsynch (experiment 1)¹

Item	1 PGF	2 PGF	Effect of PGF difference, % (P -value)
Primiparous, % (no./no.)	46.1 (41/89)	48.2 (40/83)	4.6 (0.45)
Multiparous, % (no./no.)	36.6 (37/101)	45.0 (45/100)	23.0 (0.14)
P -value	0.24	0.77	
Overall, % (no./no.)	41.1 (78/190)	46.4 (85/183)	12.9 (0.17)

¹The relative difference between treatments is calculated as the difference in P/AI between 2 PGF minus 1 PGF P/AI and then divided by the 1 PGF P/AI. All cows that were enrolled in Double-Ovsynch are included in this analysis.

second PGF on P/AI was also observed when all cows were included in the analysis ($P = 0.049$).

DISCUSSION

In experiment 1, treatment with a second PGF increased the percentage of cows with complete CL regression at the end of the Double-Ovsynch protocol from 83 to 97%. Numerous other studies have also reported that Ovsynch-type protocols that use only a single PGF treatment have 10 to 25% of cows with inadequate regression of the CL near the time of final GnRH or AI. For example, in cows resynchronized at 32d after TAI with Ovsynch (Resynch-32) only 83.7% of cows had complete CL regression (<0.4 ng/mL at 56 h after PGF), whereas cows resynchronized with Double-Ovsynch had 89.1% CL regression (Giordano et al., 2012b). In that study, the presence of a new CL had a major effect on whether the cows underwent complete CL regression as evidenced by only 79.4% regression in cows with a new CL compared with 93% CL regression in cows without a new CL (Giordano et al., 2012b). A study comparing a single treatment with dinoprost (25 mg) or cloprostenol (0.5 mg) reported that either PGF product induced complete CL regression in only about 80% of cows (80 vs. 79%; dinoprost vs. cloprostenol; Martins et al., 2011). Similarly, other studies using the 7-d Ovsynch protocol have reported between 73.9 to 95.2% of cows with complete CL regression following a single treatment with different types of PGF products in lactating dairy cows (Souza et al., 2007; Santos et al., 2010; Giordano et al., 2012a). In our study, 18% of cows did not completely regress the CL after a single PGF treatment and, in contrast to previous studies (Martins et al., 2011; Giordano et al., 2013), inadequate CL regression was similar in primiparous and multiparous cows. Further, we not only found a decrease in percentage of cows with elevated P4 (≥ 0.5 ng/mL), but we also found a change in the distribution of cows by circulating P4 at final GnRH with an increased percentage of cows with very low circulating P4 (<0.2 ng/mL) after 2 compared with one PGF treatment. It is not yet clear what cut-off should be used for evaluating the negative effect of P4 near AI on fertility with cut-offs from 0.1 to 0.5 ng/mL being supported by different analyses of P4 versus P/AI (Souza et al., 2005; Pereira et al., 2013; Wiltbank et al., 2014b). What was particularly intriguing in our analysis was that inadequate CL regression occurred in a larger percentage (33.3%) of cows with lower P4 concentrations (2.0 to 4.8 ng/mL) at the time of the single PGF treatment, compared with cows with greater circulating P4 at time of PGF (11.9%). Treatment with 2 PGF produced complete CL regression in

Figure 3. Effect of concentrations of progesterone (P4) at time of prostaglandin $F_{2\alpha}$ (PGF; for cows with P4 ≥ 2 ng/mL at first PGF) in cows receiving 1 (squares) or 2 (triangles) PGF treatments (experiment 1; $n = 176$ for 1 PGF; $n = 168$ for 2 PGF) on (A) CL regression (percentage of cows with <0.5 ng/mL at second GnRH treatment), (B) pregnant/AI for all of these cows, or (C) pregnant/AI including only the cows that underwent complete CL regression.

almost all cows, whether they had lower P4 (95.1%) or elevated P4 (97.6%) at time of first PGF treatment. It should be noted that we eliminated nonsynchronized cows (7.8%; 29/373) by using a limit of 2 ng/mL of circulating P4 at the PGF treatment. A previous study also reported that cows with low P4 at PGF were more

Table 3. Quartile analysis based on circulating progesterone concentration [P4] at time of first PGF treatment¹

Item	Quartile 1	Quartile 2	Quartile 3	Quartile 4
1 PGF				
[P4] (range)	2.0–4.8	4.9–6.7	6.8–8.7	8.8–12
CL regression, ² % (no./no.)	66.7 (28/42)	84.4 (38/45)	95.5 (42/44)	84.4 (38/45)
Pregnant/AI, % (no./no.)	31.0 (13/42)	40.0 (18/45)	47.7 (21/44)	44.4 (20/45)
2 PGF				
[P4] (range)	2.0–5.4	5.5–7.0	7.1–8.6	8.7–12
CL regression, % (no./no.)	95.1 (39/41)	95.6 (43/45)	100 (43/43)	97.4 (38/39)
Pregnant/AI, % (no./no.)	31.7 (13/41)	46.7 (21/45)	55.8 (24/43)	56.4 (22/39)

¹The percentage with CL regression and pregnant/AI are shown for cows treated with either 1 or 2 PGF treatments in experiment 1.

²Circulating progesterone <0.5 ng/mL at 56 h after first PGF in cows that had ≥2.0 ng/mL of progesterone on the day of first PGF.

Table 4. Results for effects of treatments on each individual farm in experiment 2¹

Farm	1 PGF, % (no./no.)	2 PGF, % (no./no.)	Effect of PGF difference, % (<i>P</i> -value)
CA-01	29.4 (25/85)	36.4 (32/88)	+23.64 (0.21)
CA-02	35.3 (43/122)	39.8 (45/113)	+12.99 (0.28)
CA-03	30.4 (38/125)	34.1 (47/138)	+12.03 (0.31)
NM-04	35.2 (68/193)	37.7 (66/175)	+7.04 (0.35)
NY-05	32.7 (32/98)	31.2 (34/109)	−4.47 (0.47)
NY-06	29.4 (27/92)	42.0 (42/100)	+43.11 (0.01)
PA-07	39.2 (31/79)	43.6 (27/62)	+10.98 (0.37)
TX-08	37.7 (46/122)	29.8 (37/124)	−20.86 (0.12)
WI-09	28.1 (16/57)	40.4 (23/57)	+43.75 (0.12)
WI-10	35.3 (18/51)	37.0 (20/54)	−4.94 (0.507)
WI-11	26.9 (14/52)	26.9 (14/52)	0.0 (1.0)
Total	33.3% (358/1076)	36.1% (387/1072)	+8.50 (0.068)

¹The relative difference between treatments is calculated as the difference between P/AI for 2 PGF minus 1 prostaglandin F_{2α} (PGF) divided by the 1 PGF P/AI. The *P*-values within each herd were calculated using chi-square analyses.

Table 5. Results of experiment 2 for effects of treatment and parity on percentage of cows pregnant per AI

Parity	1 PGF, % (no./no.)	2 PGF, % (no./no.)	Effect of PGF difference, % (<i>P</i> -value)
Primiparous	37.1 (99/267)	38.2 (99/259)	+3.10 (0.39)
All multiparous	32.0 (259/809)	35.4 (288/813)	+10.65 (0.07)
<i>P</i> -value	0.128	0.414	
Second and third parity	32.7 (194/593)	37.4 (220/589)	+14.19 (0.047)
Fourth or greater parity	30.1 (65/216)	30.4 (68/224)	+0.09 (0.52)

Table 6. Effect of treatment with a second prostaglandin F_{2α} (PGF) on P/AI during the Ovsynch (experiment 2) or Double-Ovsynch (experiment 1) protocols¹

Parity	1 PGF, % (no./no.)	2 PGF, % (no./no.)	Effect of PGF difference, % (<i>P</i> -value)
Primiparous	39.3 (140/356)	40.6 (139/342)	+ 3.31% (0.39)
Multiparous	32.5 (296/910)	36.5 (333/913)	+12.31% (0.043)
<i>P</i> -value	0.04	0.17	
Overall	34.4 (436/1266)	37.6 (471/1251)	+9.45% (0.049)

¹Results from experiments 1 and 2 were combined for the analysis with all cows assigned to the experiments included in the analysis.

likely to not have complete CL regression (Martins et al., 2011). This previous study did not eliminate cows that were below 2 ng/mL at the time of PGF, used both cloprostenol or dinoprost as the PGF source, and measured P4 on 3 separate occasions to determine complete CL regression (56, 72, and 96 h after PGF). Based on results from both these studies, it seems clear that the largest problem with complete CL regression is due to cows that have low P4 at the time of PGF treatment, although even cows with higher P4 have more than 10% of cows with inadequate CL regression (Table 3). The reason that cows with lower P4 have a relative resistance to PGF action is not clear at this time but may indicate that younger CL or fewer CL were present in these cows. Because cows ovulate about 28 h after GnRH treatment (Pursley et al., 1995), the cows that ovulate to the GnRH treatments given 14 and 7 d before PGF treatment would be expected to have d 13 and d 6 CL at the time of PGF. In a previous study (Giordano et al., 2012b), cows treated with a single PGF were much more likely to not have complete CL regression, if cows had a single d 6 CL (35.6% regression) than if they had a single d 13 CL (3.0%), or a d 6 and a d 13 CL (8.2%). Thus, regression of the d 13 CL may be enhancing regression of the d 6 CL, possibly due to a more pronounced decline in circulating P4 and resulting release of PGF from the uterus. Cows with lower P4 in our study or in the previous study (Martins et al., 2011) may not have had as large of a decline in circulating P4 and consequently no augmentation of luteolysis from the uterus. Definitive testing of this speculative idea or other potential reasons for lack of complete CL regression will require future research.

Experiment 1 was not powered for detection of fertility effects due to the second PGF. For cows given either 1 or 2 PGF treatments, fertility increased with increasing circulating P4 at the time of PGF treatment. This relationship has been previously reported for cows given only a single PGF treatment (Martins et al., 2011). However, in our study, this relationship was particularly clear in cows treated with 2 PGF ($P = 0.02$), and this has not been previously reported. Of particular interest, the second PGF only appeared to enhance fertility in cows with elevated P4 and not in cows that had low P4 at the first PGF treatment. This is puzzling because the greatest improvement in CL regression caused by the second PGF treatment was found in the cows with low P4 at the time of PGF treatment. This intriguing result will need to be replicated in future studies but suggests that the second PGF may be enhancing fertility by effects other than decreasing percentage of cows with inadequate luteolysis at 56 h after PGF. Perhaps an earlier time to low P4 or increased circulating estradiol may underlie

the observed improvements in fertility produced by a second PGF treatment (Brusveen et al., 2009; Martins et al., 2011). Changes in circulating P4 or E2 could increase fertility by improving oocyte quality through optimized follicle development and size, could improve gamete transport in the uterus and oviduct, potentially leading to improved fertilization, or could alter uterine environment in a way that improves embryonic development, rescue of the CL, or attachment/implantation of the developing embryo (Pursley and Martins, 2011; Wiltbank et al., 2014a).

Experiment 2 represents one of the larger multi-site studies undertaken to examine different reproductive management protocols. Eleven different herds were found in the West, Southwest, Midwest, and Northeast regions of the United States. No effect of farm was found from the logistic regression analysis, although as shown in Table 4, treatment effects ranged from a significant positive effect ($P = 0.01$) to a tendency for a negative effect ($P = 0.12$) on individual farms. When all results were combined, about 3% absolute improvement or 8.5% relative improvement was found in fertility due to the second PGF treatment. Nonetheless, even with more than 2,100 cows in this experiment, the fertility enhancement was only a statistical tendency ($P = 0.068$). As in experiment 1, the fertility enhancement was only observed in multiparous ($P = 0.073$) and not in primiparous ($P = 0.39$) cows. Further separation of the parity groups showed that the fertility enhancement was only present in second and third lactation cows ($P = 0.047$) and not in fourth or greater lactation cows ($P = 0.517$). Greater fertility in primiparous than multiparous cows has been previously reported in numerous studies using Ovsynch-type protocols (Herlihy et al., 2012; Giordano et al., 2013; Wiltbank and Pursley, 2014); however, this unique relationship between parity and response to 2 PGF has not been previously observed. Further experiments are required to confirm this relationship.

The final combined analysis of both experiments provided the most statistical power to test our main hypothesis (Table 6). Overall, P/AI increased along with a 9.45% relative increase in fertility. As in both experiments, the effect was only statistically detectable in multiparous ($P = 0.043$) and not in primiparous ($P = 0.39$) cows. In our study, an effect of parity on fertility was only detected in cows treated with 1 PGF ($P = 0.04$) and not in cows treated with 2 PGF ($P = 0.17$). Future studies should directly test whether parity effects with Ovsynch-type protocols are related primarily to differences in CL regression between parities or reflect other fertility differences related to parity.

An important basic biological question is why complete CL regression did not occur after a single PGF

treatment in some cows. Lack of complete CL regression in response to a single PGF treatment has been termed lack of luteolytic capacity (Tsai and Wiltbank, 1998; Diaz et al., 2002) and is observed more frequently in younger CL in many species including cattle, sheep, pigs, horses, rabbits, marmosets, and humans (Diaz et al., 2000; Wiltbank et al., 2012; Smith and Meidan, 2014). Comparisons of cellular and molecular responses to PGF in CL with or without luteolytic capacity have indicated that PGF invokes some but not all of the biological responses in younger compared with older CL (Tsai and Wiltbank, 1998; Mondal et al., 2011). Receptors for PGF are present primarily on large luteal cells (Wiltbank, 1994; Anderson et al., 2001) but in CL either with or without luteolytic capacity (Wiltbank et al., 1995; Diaz et al., 2000, 2002). Treatment with PGF on d4 of the bovine estrous cycle (CL without luteolytic capacity) elicited a similar change in expression of many genes in the early CL (~25%) as were elicited by PGF treatment of older CL (Mondal et al., 2011). However, some key gene expression changes were not produced by PGF in CL lacking luteolytic capacity including: increased mRNA for genes involved in intraluteal PGF production (Tsai and Wiltbank, 1998) and immune responses (Tsai et al., 1997; Mondal et al., 2011) or decreased mRNA for genes involved in steroidogenesis (Tsai and Wiltbank, 1998; Diaz and Wiltbank, 2005) and angiogenesis (Mondal et al., 2011; Zalman et al., 2012). Thus, PGF-induced intra-cellular signaling pathway(s) are altered in CL without luteolytic capacity, although the definitive lesion is still under investigation. A recent study using the porcine CL indicated a lesion in PGF induction of c-jun and jun-D in CL without luteolytic capacity (Diaz et al., 2013), which would prevent activation of the AP-1 transcriptional complex that is central to luteolytic responses (Chen et al., 2001; Davis and Rueda, 2002). In addition, inhibition of intraluteal P4 production, using epostane before PGF treatment, led to recovery of luteolytic responses to PGF in CL without luteolytic capacity (Diaz et al., 2011), suggesting that maintenance of high intraluteal P4 production may be central to lack of luteolytic capacity. Obviously, future basic biological research is needed to completely define the intra- and intercellular pathways that must be overcome to allow treatment with a single PGF to induce complete CL regression and optimal fertility in dairy cows.

From a practical standpoint, treatment with a second PGF during Ovsynch produced about 10% more pregnancies. Although a single PGF is fairly inexpensive in the United States, a complete economic analysis is needed to determine whether the reduction in semen costs due to improved conception risk, reduction in fu-

ture costs for detection of estrus or for synchronization of nonpregnant cows, and increased value from the earlier pregnancy offset the increase in hormonal costs and labor that are required with this procedure (Giordano et al., 2011). In particular, the economic analysis needs to evaluate the value of a second PGF for multiparous separate from an analysis for primiparous cows because such a clear difference was present between parities in the response to this treatment. The results that were observed using the circulating P4 concentrations indicate that future research may allow even more precise targeting of cows that are most likely to benefit from the second PGF treatment.

CONCLUSIONS

Treatment with a second PGF during the Double-Ovsynch protocol reduced the percentage of cows with inadequate regression of the CL at the time of the final GnRH treatment. An increase was observed in fertility with about 10% more pregnancies produced in cows treated with the second PGF during an Ovsynch or Double-Ovsynch protocol, making this likely to be a practical treatment for many dairy farms. Future studies are needed to understand physiological conditions that underlie the improvements in CL regression and P/AI with this treatment.

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