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Evaluation of Hormone Treatments in a Modified Ovulation Synchronization Protocol in Dairy Heifers

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Abstract: Ov-Synch is a successful ovulation synchronization protocol when used in lactating dairy cows, with pregnancy rates equaling those seen in animals bred at detected estrus and is now accepted as the industry standard protocol. However, when used in dairy heifers, current protocols do not achieve the same success as seen in dairy cows. Our objectives in this study were to: determine the most effective hormone treatment intervals for the administration of Gonadotropin Releasing Hormone (GnRH) and Prostaglandin $F_{2\alpha}$ (PG) to induce and synchronize ovulation of the dominant follicle in dairy heifers and) assess the influence of the different hormone treatment intervals on subsequent fertility in response to timed-AI. Heifers (n = 60) were synchronized on d 0 with a single GnRH injection (Cystorelin; 100 μg) and then divided into two groups. Heifers were treated with PG (Lutalyse; 25 mg) either on day 7 (n = 30) or day 12 (n = 30) followed by a second dose of GnRH (100 μg) 48 h later. All heifers were bred by AI 16 h after the last GnRH injection. Ultrasonography was performed daily on a sub-population of heifers (n = 9/group) to monitor follicular dynamics and blood was collected from an additional sub-population of heifers (n = 6/group) for serum LH analysis. Blood was collected from all heifers on days 3, 6, 12, 21 and 45 post-AI for serum Progesterone (P_4) analysis. Peak concentrations of Luteinizing Hormone (LH) obtained 60 min after GnRH treatment did not different ($p>0.05$) between day 7 vs. day 12 heifers (15.3±1.9 and 13.5±2.2 ng mL^{-1}, respectively). Total LH concentrations (0-16h post-second GnRH treatment) were 49.5±5.7 and 47.0±8.3 ng mL^{-1}. Mean dominant preovulatory follicle size on day of second GnRH injection was 15.1±1.9 and 17.0±1.8 mm and did not differ ($p>0.05$) between 7 and 12 day heifers, respectively. Compared with day 12 heifers, day 7 heifers had higher pregnancy rates (10% vs. 27%, respectively) and a reduced incidence of behavioral estrus (87.5% vs 58.8%), respectively) within 18 days post-insemination. Of the day 12 heifers scanned, 44.4% (4/9) had persistent follicles. In addition, serum concentrations of P_4 21 days post-AI were higher ($p<0.05$; 4.8 vs. 0.7 ng mL^{-1}) for day 12 than for day 7 non-pregnant heifers. These data suggest that extending the interval between the initial GnRH and PG injections (12 vs. 7 days) failed to reduce the variability in response of dairy heifers to synchronization of ovulation.

Key words: Dairy heifers, ovulation synchronization, gonadotropin releasing hormone, prostaglandin $F_{2\alpha}$

INTRODUCTION

With increased knowledge of the bovine estrous cycle and the introduction of the use of artificial insemination in the dairy industry, efforts have intensified to control the emergence of estrus and the timing of ovulation, while simultaneously trying to minimize the problem of inaccurate and unreliable estrus detection. Synchronization of ovulation is a commonly used practice in the dairy industry today and a plethora of protocols using exogenous hormones have been developed to manipulate the reproductive cycle in cattle. Such protocols allow producers to have more precise control over the reproductive status of their animals so they may take advantage of seasonal and price variations of milk (Risco *et al*., 1998). When used with timed Artificial Insemination (AI), synchronization of ovulation reduces labor by eliminating the need for estrus detection (Pursley *et al*., 1997a). However, the steady increase in average milk production per cow per year has been associated with declines in estrus detection rate, increase in days open and a decrease in conception rates

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(Pursley *et al*., 1997b) has led to the need for improved protocols. In addition, the desire to introduce heifers at a younger age into the dairy herd has increased demand for protocols that will facilitate their introduction.

The most commonly used protocol today is Ov-Synch. The protocol utilizes Gonadotropin Releasing Hormone (GnRH) and Prostaglandin $F_{2\alpha}$ (PG) given at a seven day interval which is followed by a second treatment of GnRH two days later and timed AI 16 h after the second GnRH injection. This protocol, when used with timed AI, is an excellent management tool for the dairy industry. It has become an effective way of managing reproduction because it eliminates the need for estrus detection and works well in lactating dairy cows. However, pregnancy rates of lactating cows are not different between those bred using Ov-Synch and those bred at estrus (38.9 and 37.8%, respectively) (Lucy, 2001). In contrast, heifers have an average pregnancy rate of 74.4% when bred at estrus compared to 35.1% when bred using the Ov-Synch protocol (Lucy, 2001; Lee *et al*., 1983). These pregnancy rates are unacceptably low for heifers. While, there is some conflicting evidence, (Schmitt *et al*.,1996) most researchers do not recommend the use of Ov-Synch in heifers (Pursley *et al*., 1997a; Thatcher *et al*., 1983; Drost *et al*., 1999). However, by modifying the current protocol to more closely match ovarian follicular activity in heifers, a similar success rate might be observed.

One factor that may explain the better results obtained with Ov-Synch in lactating cows compared with heifers is the difference in the number of follicular waves per estrous cycle. Research has shown that heifers predominantly have three waves per cycle (Savio *et al*., 1988; Sirois and Fortune, 1988; Rajamahendran and Taylor, 1991) while cows tend to have two follicular waves per estrous cycle (Lucy, 2001). Ov-Synch is dependent on the synchrony of the corpus luteum and the dominant follicle (Lucy, 2001). Previous studies have shown that luteal development was not synchronized in heifers when the Ov-Synch protocol was used (Lucy, 2001). The protocol was developed with a two follicular wave cycle in mind. Because heifers tend to have 3 waves rather than 2 per estrous cycle, the injection intervals may not be appropriate and may be interacting with the follicular wave at a time when it is not as responsive to hormone treatment. By lengthening the injection interval between GnRH and PG, it may be possible to catch the second follicular wave of the cycle during the growth phase of the dominant follicles, therefore causing ovulation. This would increase synchrony and conception rates to the Ov-Synch protocol.

Therefore, the major objectives of this study were: to determine the most effective injection intervals for the administration of GnRH and PG to induce and synchronize ovulation of the dominant follicle in dairy heifers and to assess the influence of GnRH -PG injection intervals (7-day vs 12-day) on subsequent fertility in response to timed AI.

MATERIALS AND METHODS

Animal handling and protocol: Sexually mature, nulliparous Holstein heifers (n = 60; 364.2±7.2 kg), obtained from the Bearden Dairy Research Center at Mississippi State University, were randomLy assigned to one of two treatment groups, 7-day (n = 30; 7 day GnRH-PG injection interval) and 12-day (n = 30; 12 day GnRH-PG injection interval). Heifers assigned to the 7-day treatment group (7-day) followed the standard Ov-Synch protocol, receiving a 100 mg i.m. injection of Cystorelin® (Merial, Ltd., Islin, NJ; GnRH) on day -9, followed by a 25 mg i.m. injection of Lutalyse® (The Upjohn Company, Kalamazoo, MI; PG.) on day -2 and a second injection of GnRH (100 μg i.m.) on day 0 (day of breeding; Fig. 1). Heifers assigned to the 12-day treatment group (12-day) followed a modified Ov-Synch protocol (Fig. 1). The 12-day heifers received their initial injection of GnRH (100 μg i.m.) on day -14 followed by an injection of PG (25 mg i.m.) on day -2 and a second injection of GnRH (100 μg i.m.) on day 0. All heifers were bred 16 h following the second GnRH injection and estrus was observed twice daily from Day 0 to Day 24 post-AI. All injections were administered at 06:00 am. The hormone treatments were performed during the summer month of August.

Reproductive management and ultrasonography: Ultrasonography was performed daily (06:00) on a subset (randomLy chosen using a random number table) of heifers (different from the LH subsample) from each treatment group (n = 9/group) using an Aloka 500 V ultrasound fitted with a 5.0 MHz transducer (Aloka Co. Ltd., Wallingford, CT, USA). The 18 heifers were scanned from the day of the first GnRH injection through day of breeding and every 12 h for 48 h or until ovulation (which ever came first) starting the day of breeding. The largest and subordinate follicles were scanned and measurements recorded daily. All animals were checked for pregnancy via ultrasound on day 21 at day 45 post-AI. Estrus activity was observed twice daily from day of breeding to 45 days post-breeding.

Blood sampling and hormone analysis: Blood samples were collected from 60 heifers by jugular venipuncture for

serum Progesterone (P_4) analysis on days 0, 3, 6, 12, 21 and 45 post-AI. Blood samples were refrigerated for at least 4 h before centrifugation and separation of serum. Serum was stored at -20°C until assayed for P_4 by Radioimmunoassay (RIA). Blood samples were also collected for serum Luteinizing Hormone (LH) by RIA at 0, 0.5, 1, 2, 4, 6, 8 and 16 h following the second GnRH injection from a subset of heifers in each treatment group (n = 6/group). The blood was collected and processed as mentioned above. The subset of heifers for LH sampling and analysis were randomLy selected using a random number table.

A P_4 radioimmunoassay was performed using the DSL-3900 Active™ Progesterone Kit (Diagnostic Systems Laboratories, Inc. Webster, TX), following the instructions and procedures as described by the manufacturers. The intra- and inter-assay coefficients of variation were 5.40 and 9.97%, respectively. Serum LH concentrations were performed by Dr. S. Whisnant (North Carolina State University) and were determined as described by Schoppee *et al.* (1996) with the following modifications. Bovine LH (bLH) for iodination and standards was obtained from the National Hormone and Pituitary Program. Briefly, 200 μL of serum or standard were added to tubes with 300 μL of 0.01 M phosphate buffered saline (pH 7.0, containing 0.1% gelatin; PBS-GEL) followed by the addition of 200 μL of anti-bLH antiserum (#190, courtesy of T.E. Kiser, University of Georgia), diluted 1:80,000 with PBS containing 0.05 M disodium EDTA and 1: 300 normal rabbit serum (PBS-EDTA) then incubated for 24 h. On the next day 100 μL of ^{125}I labeled bLH diluted to 30,000 cpm in PBS-GEL was added to all tubes and the assay incubated for another 24 h. Next 200 μL of a sheep anti-rabbit serum (secondary antibody NCSU #91 diluted 1:16 in PBS-EDTA) was added and tubes were incubated for a further 24 h. To terminate the assay, 1 μL PBS with 6% polyethylene glycol (Fisher Scientific, Raleigh, NC) was added to all except total count tubes and incubated 1 h at 4°C. The tubes were centrifuged at 1700 X g for 30 min. The supernatant was poured off and tubes air dried before being counted in a Cobra auto gamma counter (Perkin Elmer, Life and Analytical Sciences, Shelton, CT). All incubations were performed at room temperature unless otherwise stated. The sensitivity of the assay was 0.3 ng mL^{-1} and the intra- and inter-assay coefficients of variation were 7.1 and 10.9%, respectively.

Statistical analysis: Statistical analysis was performed using the GLM procedure of SAS® and StatView (SAS Institute, Cary, North Carolina). The experimental design was a completely randomized design with repeated measures. The study consisted of two treatment groups (7 and 12-day). The model utilized included treatment, heifer within treatment, sampling time and sampling time by treatment interaction. Treatment effects were tested against heifer within treatment variation. Differences were considered significant when a probability of 0.05 or less was obtained and tendencies were determined when probability was between 0.05 and 0.10.

RESULTS

Follicular dynamics: Ultrasound measurements revealed that the size of the largest follicle on day of PG administration was greater ($p<0.05$) in the 12-day heifers (13.2±1.64 mm for 7-Day heifers, n = 9 and 17.2±1.21 for 12-Day heifers, n = 8) but that follicle size on day of first GnRH treatment did not differ ($p>0.05$) between treatment groups (11.61±0.96 and 13.2±1.21 mm for 7 and 12-Day heifers, respectively). Mean dominant preovulatory follicle size after the second GnRH injection also did not differ ($p>0.05$) between 7 and 12-day heifers (15.1±1.9 and 17.0±1.8 mm, respectively). Moreover, size of the largest follicle did not differ ($p>0.05$) between day of PG and day of GnRH treatment, irrespective of treatment group (PG: 12.9±0.91 and GnRH: 3.9±1.28 mm). An increased incidence of persistent follicles was observed in the 12-day heifers. Of the 9 heifers that were examined in this group by ultrasound, 4 (44.4%) exhibited persistent follicles with a mean size of 18.4±4.6 and 19.9±3.9 mm following PG and second GnRH treatments, respectively and 17.7±3.4 mm 48 h post-second GnRH treatment. No persistent follicles were observed in the 7-day group of heifers. Persistent follicles were determined to be lutenized follicles that did not ovulate by 48 h post-AI as described by Twagiramungu *et al.* (1995).

Hormone analysis: Non-pregnant heifers in the 12-day treatment group (n = 27) had significantly higher ($p<0.05$) concentrations of P_4 on days 0, 3 and 6 post-AI

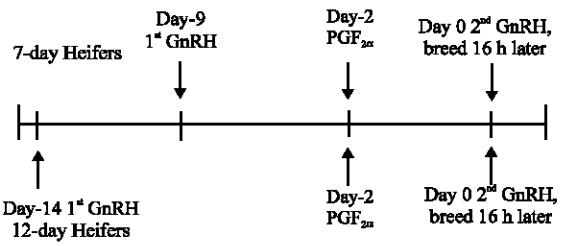

Fig. 1: Treatment timeline for the Ov-Synch and Modified Ov-Synch protocol used in 7 and 12-day heifers

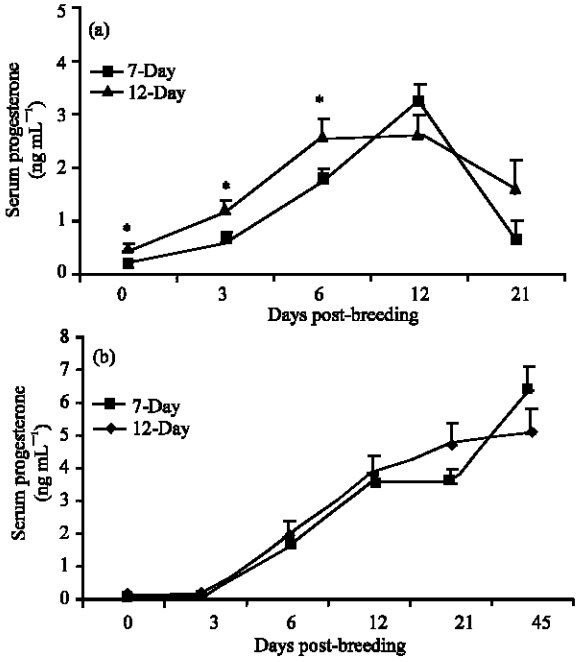

Fig. 2: Serum concentrations of progesterone in non-pregnant and pregnant heifers. Values for P4 were significantly (* = $p<0.05$) higher in the 12-Day (n = 27) than 7-Day (n = 22) non-pregnant heifers on Day 0, 3 and 6 post AI (panel A). Day 0 = day of breeding (AI). There was no difference ($p<0.05$) in P4 values between 7-Day (n = 8) and 12-Day (n = 3) pregnant heifers (panel B). Day 0 = day of breeding (AI)

(0.43±0.09, 1.14±0.23 and 2.58±0.33 ng mL^{-1}, respectively) than those non-pregnant 7-day heifers (n = 22; 0.16±0.050, 0.56±0.14 and 1.74±ng mL^{-1}, respectively). Serum P_4 concentrations for both 7 and 12-day treatment groups were above 1.0 ng mL^{-1} by day 6 post-AI and values remained similar through Days 12 and 21 post-AI (Fig. 2a). There were no significant differences ($p>0.05$) in P_4 concentrations among pregnant heifers, between treatment groups (n = 8 for 7-day, n = 3 for 12-day; Fig. 2b). Serum LH concentrations and response did not differ ($p> 0.05$) between the 7 and 12-day treatment groups following the second GnRH injection (Fig. 3). Mean LH peak was observed at 60 min post GnRH (Fig. 3) and there were no significant differences ($p>0.05$) between observedgroups. Table 1 displays the characteristics of the LH response in each treatment group.

Estrus detection: Estrus was observed twice daily from the day of breeding until 45 days post-breeding. A noticeable shift was observed in the number of days for return to first estrus post-AI. The average days to estrus

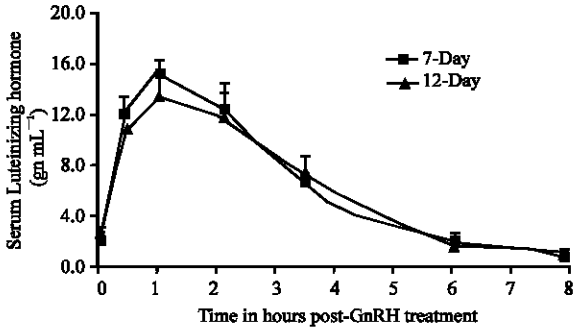

Fig. 3: Luteinizing hormone response to the second GnRH injection administered on Day 0 as measured in serum. The response to GnRH stimulation was not different ($p>0.05$) between 7 and 12-Day heifers

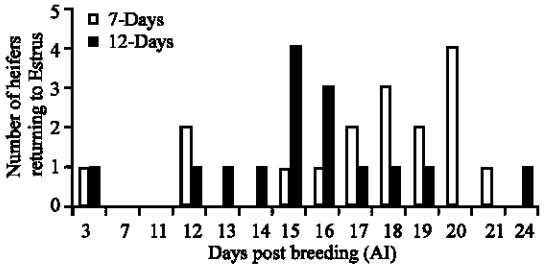

Fig. 4: Number of heifers returning to first estrus between days 12 and 21 post-AI. One heifer in each group returned to estrus on day 3 and one 12-day heifer returned to estrus on Day 24 post-AI giving a total of seventeen 7-Day and fifteen 12-Day heifers returning to estrus

Table 1: Luteinizing hormone response characteristics following the second GnRH injection

	Heifer treatment		
Measures of LH	7-Day (n = 6)	12-Day (n = 6)	p-value
Peak LH (ng mL^{-1})	17.2±1.22	14.27±4.49	0.20
Time to LH peak (min)	55.0±14.32	50.0±6.33	0.76
Overall LH (ng mL^{-1})	6.18±0.72	5.87±0.81	0.81

interval (first estrus, Day 12-21 post-AI) for 7-day heifers was longer ($p<0.05$) than for 12-day heifers (n = 16, 17.6±0.70 and n = 13, 15.5±0.53 days respectively; Fig. 4). Of all the 7 and 12-day heifers returning to estrus 41.2% (7/17) did so before Day 18 post-AI while 58.8% (10/17) returned 18-24 days post-AI. In contrast, of all the 12-day heifers returning to estrus 80% (12/15) returned <18 days post-AI while 20% (3/15) returned 18-24 days post-AI. Pregnancy rates were 27% (8/30) and 10% (3/30) for the 7-day and 12-day heifers, respectively.

DISCUSSION

This study demonstrated that increasing the GnRH-PG injection interval from 7-12 days in the Ov-Synch protocol did not increase pregnancy rates in heifers. While we observed no differences between treatment groups in follicle size, LH characteristics, or P_4 concentrations in pregnant heifers, we did find differences in time to first estrus and P_4 concentrations among non-pregnant heifers.

Persistant follicles were observed in 44% of the 12-day heifers. While not all 30 animals were scanned, P_4 data indicated that more than 4 heifers from the 12-day treatment group may have exhibited persistent follicles. The persistent follicles were thick-walled, P_4 secreting follicular cysts. The significantly higher concentrations of P_4 observed in the non-pregnant 12-day treatment group on days 0, 3 and 6 post-AI supports our findings of persistent follicles among 12-day heifers. One reason why this may have occured is suggested by Twagiramungu and colleagues (Twagiramungu *et al*., 1995) who state that the use of GnRH and PG for ovulation synchronization gives the dominant follicle two options, either ovulate or persist. For a follicle to ovulate, luteolysis must be complete with estrogen concentrations decreasing after ovulation occurs (Twagiramungu *et al*., 1995). If PG-induced luteolysis is incomplete then estrus and ovulation do not occur (Twagiramungu *et al*., 1995). Prolonged sub-luteal concentrations of P_4 in the blood appear to increase frequencies of pulsatile LH and inhibit the preovulatory LH surge, thereby preventing ovulation and allowing the dominant follicle to develop into a persistent follicle (Roberson *et al*., 1989; Savio *et al*., 1993; Stock and Fortune, 1993; Scnchez *et al*., 1995).

As stated above, ovulation of the dominant follicle following treatment depends upon the degree of induced luteolysis (Twagiramungu *et al*., 1994, 1995). When luteolysis is complete, P_4 concentrations are below 1.0 ng mL^{-1}. When luteolysis is incomplete, P_4 concentrations are above 2.0 ng mL^{-1} and estrus will not occur. As such, the dominant follicle does not ovulate; it becomes persistent Barros *et al*. (2000). Several studies have shown that subluteal concentrations of P_4 for a prolonged period can cause persistent follicles (Barros *et al*., 2000). Heifers that exhibit persistent follicles cannot become pregnant until those follicles ovulate or degenerate.

One explanation of why the heifers in this study treated at a 12-day interval exhibited such low pregnancy rates is that when the second GnRH injection was administered, the dominant follicle was already on its predetermined path of atresia. Once a dominant follicle has been determined to become atretic, it can no longer be an ovulatory follicle and treatment will have little effect (Twagiramungu *et al*., 1995). If treatment is administered at this time, incomplete luteolysis may occur. The follicle is then lutenized and becomes persistent (Twagiramungu *et al*., 1995). Failure of persistent follicles to regress reduces the chances of success. This may explain the persistent follicles and low conception rates seen in the 12-day treatment group.

Research conducted by Pursley *et al*. (1995, 1997) has shown that cows appear to have a functionally dominant follicle which is responsive to LH for a greater proportion of the estrous cycle than heifers. Their data also have shown that folliclular growth appears to be more rapid and the length of the follicular wave appears to be longer in heifers than in cows (unpublished data) (Pursley *et al*., 1995, 1997). They further state that heifers that do not respond to the first GnRH injection would likely be in the first three days of a follicular wave, when PG is given (traditionally seven days later) these heifers would be on day nine or ten after the initiation of a follicular wave. This would be a time when the old follicle would lose dominance and a new follicular wave would be emerging. The old follicle would be regressing and unresponsive and the new dominant follicle would be too immature to respond to the PG treatment. With this said, it is possible that our choice of a 12-day interval was a little too late and an interval shorter than seven days would be more appropriate. This may result in a better synchrony of follicular growth and ovulation in the heifer.

Estrus activity was observed twice daily from the day of AI until 45 days post-AI. The data showed that 12-day heifers exhibited short cycles (less than 18 days), while the 7-day treatment group exhibited normal estrous cycles (18-21 days). There was a distinct shift in estrous cycle length, which may be due to the persistent follicles and/or the lack of synchrony the 12-day heifers exhibited. As reported earlier, persistent follicles are due to incomplete luteolysis of the dominant follicle (Twagiramungu *et al*., 1995). The dominant follicle does not ovulate, but persists, preventing the animal from coming into estrus. Once the follicle regresses or ovulates, the animal will exhibit estrus. Therefore, the 12-day heifers would not have exhibited estrus at the time of AI, but would have exhibited estrus later, once the follicle regressed. This might explain why the 12-day heifers came into estrus prior to 18 days post-AI.

In summary, altering the injection interval in the Ov-Synch protocol from 7-12 days was not an optimal time. It appears that combinatorial protocols that employ the use of controlled internal drug-release devices (i.e., CIDRS) with the standard Ov-Synch protocol may prove to be more beneficial approach to in increasing pregnancy rates and embryo survival in dairy cattle (El-zarkouny *et al*., 2004; Kim *et al*., 2005).

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