Hot topic: Successful fixed-time insemination within 21 d after first insemination by combining chemical pregnancy diagnosis on d 18 with a rapid resynchronization program

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ABSTRACT

Cattle that are not pregnant to first fixed-time artificial insemination (TAI) may be resynchronized for a second TAI if they are found nonpregnant at pregnancy diagnosis. The specific interval between first and second TAI ranges from 4 to 8 wk. The selected interval depends on the available method of pregnancy diagnosis and the efficiency of the resynchronization program. The objective of this experiment was to evaluate a pregnancy diagnosis and resynchronization system that achieved a 21-d interval between TAI. This 21-d interval approximates the natural return-to-service interval. It also enables resynchronization to be implemented within the same estrous cycle in which cattle are first inseminated. Holstein heifers were randomly assigned to a 21-d resynchronization program (21d_resynch; $n = 40$) or a control group in which estrus was observed for the purpose of re-insemination (control; $n = 29$). The 21d_resynch heifers were diagnosed for pregnancy on d 18 after a TAI (d 0) by using predetermined cut-off values for 2'-5' oligoadenylate synthetase 1 (Oas1) gene expression in leukocytes and plasma progesterone concentration. Heifers that were not pregnant to first TAI had greater expression of Oas1 at the time of PGF_{2α} (d -3) than pregnant heifers, but this relationship was reversed on d 18 after TAI: the heifers that were pregnant to first TAI had almost 5-fold greater expression of Oas1 compared with nonpregnant heifers. Nonpregnant heifers in the 21d_resynch group were injected with a luteolytic dose of PGF_{2α} on d 19 and were injected with GnRH on d 21 and submitted to TAI. The pregnancy per AI after first insemination was similar for 21d_resynch (50.0%; pregnancy diagnosis on d 18) and control (51.7%; pregnancy diagnosis on d 27). Likewise, no difference was detected in second insemination pregnancy per AI for 21d_resynch (36.8%; nonpregnant heifers TAI on d 21) and control (35.7%; nonpregnant heifers inseminated at return to estrus

or after nonpregnant diagnosis on d 27). The interval between first and second insemination was shorter for 21d_resynch compared with control (21.0 ± 0 and 27.5 ± 2.1 d). The conclusion is that a TAI resynchronization can be programmed within 21 d of previous TAI when a d 18 pregnancy test and a rapid resynchronization are used.

Key words: Oas1, pregnancy, resynchronization, heifer

A reproductive management program was proposed by Lucy et al. (2004) that employed pregnancy detection at ≤ 18 d after fixed-time artificial insemination (TAI) and programmed re-insemination every 21 d. Within this system, nonpregnant cattle are synchronized for second and all subsequent TAI at an interval that approximates a naturally occurring estrous cycle (21 d). The enabling aspects of this system are that cattle theoretically have a corpus luteum (CL) and a dominant follicle on d 18 of the estrous cycle (assuming a prototypical 2 follicular wave estrous cycle; Lucy, 2007). The CL has achieved full luteolytic capacity to PGF_{2α} (Lauderdale, 1972) and the dominant follicle is large enough to ovulate in response to a timed GnRH injection given a few days after PGF_{2α} injection. One week later (e.g., d 25 after first insemination) similar ovarian structures may not exist because in the nonpregnant cattle that were not observed in estrus and are eligible for resynchronization the CL has likely regressed and the cattle ovulated. The difficulties experienced with resynchronization during this time (4 wk after TAI; Fricke et al., 2003) probably arise because the CL is newly formed (and not responsive to PGF_{2α}) and the dominant follicle is too small to ovulate in response to GnRH. If the cow is not seen in estrus, therefore, resynchronization with PGF_{2α} and GnRH is inefficient when a re-insemination interval of 4 wk is used.

Despite its advantages, pregnancy diagnosis on d 18 and subsequent rapid resynchronization for TAI on d 21 has never been tested, perhaps because a method of pregnancy diagnosis that could be used on d 18 has not been fully validated. We recently completed a series of studies in which the expression of interferon-stimulated

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Figure 1. Experimental timeline for the trial. Heifers were synchronized for fixed-time AI (TAI) and inseminated on d 0. Heifers in the 21 d resynchronization group (21d_resynch) underwent pregnancy diagnosis (PD) on d 18 by testing for 2'-5' oligoadenylate synthetase 1 (Oas1) and progesterone. Heifers diagnosed not pregnant on d 18 were treated with a luteolytic dose of PGF_{2α} (P) on d 19 and then injected with GnRH immediately preceding TAI on d 21 (G/AI). Control heifers were TAI on d 0 and observed for estrus and AI until pregnancy diagnosis by transrectal ultrasonography (TU). A preliminary (d 27) and final (d 60–62) pregnancy diagnosis was done.

gene (ISG) mRNA in peripheral leukocytes could be used to detect pregnancy in dairy heifers (Green et al., 2010). Specific ISG (e.g., Mx2 and ISG15) have shown increased transcription in peripheral leukocytes in response to interferon- τ (Gifford et al., 2007). One such ISG (2'-5' oligoadenylate synthetase 1, **Oas1**) was found to be suitable for pregnancy diagnosis on d 18 after AI in dairy heifers (Green et al., 2010). Specifically, a ratio of leukocyte mRNA for Oas1 before (d -3) and after (d 18) insemination had a 100% true-positive rate and a 0% false-positive rate for a pregnancy test. The objective of this experiment was to evaluate a pregnancy diagnosis and resynchronization system that achieved a 21-d interval between TAI.

Holstein heifers ($n = 69$) were housed at the University of Missouri Foremost dairy farm. Heifers were synchronized for first TAI with the Show-Me-Synch protocol [intravaginal insertion of progesterone-containing controlled internal drug release (CIDR) device (Pfizer, New York, NY) on d -33; CIDR device removed on d -19 (14 d after insertion); PGF_{2α} (25 mg of Lutalyse, i.m.; Pfizer) injected on d -3 (16 d after CIDR removal); GnRH (100 μ g of Cystorelin, i.m.; Merial, Duluth, GA) injected 66 h after PGF_{2α}; TAI immediately after the GnRH injection (d 0)] and inseminated with sex-selected semen (Select Sires, Plain City, OH). Blood samples were collected from all heifers on trial d -3 (immediately preceding PGF_{2α} injection) and 18 (18 d after TAI; Figure 1). Blood was collected from the median caudal vein or artery into tubes containing 100 μ L of a 15% solution of EDTA (K₃) (Tyco Healthcare Group, Mansfield, MA). The blood was placed on ice and the RNA isolation from leukocytes began within 2 h of blood collection. The RNA was extracted and purified by using the QIAamp RNA blood kit (Qiagen; Austin, TX) and subjected to DNase treatment according to the manufacturer's instructions. Purified RNA was dissolved in sterile water and its concentration was

determined by measuring absorbance at 260 nm. The purity of RNA was determined by calculating the ratio of absorbencies at 260 and 280 nm using a ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). One microgram of RNA was electrophoresed through a 1% agarose gel in Tris-borate/EDTA buffer (0.09 M Tris-borate and 0.002 M EDTA) with ethidium bromide (0.5 μ g/mL) to verify the integrity of each sample. Total RNA from leukocytes was transcribed into cDNA by using the SuperScript First Strand RT-PCR Kit (Invitrogen, Carlsbad, CA). The reverse transcription-PCR reactions were carried out using a cDNA equivalent of 25 ng of total RNA. The cDNA was amplified in an ABI Prism 7500 thermocycler (Applied Biosystems, Foster City, CA) using the QuantiTect Sybr Green PCR Kit (Qiagen) and specified primers for Oas1 [forward 5'-ACCCTCTC-CAGGAATCCAGT-3' and reverse 5'-GATTCTGGTC-CCAGGTCTGA-3']. Each 96-well PCR plate contained a no-template control (water substituted for cDNA in the reaction) to ensure there was no background contamination. Samples were assayed in duplicate. Gene expression data were expressed as fold change relative to a standard control sample that was run within each assay plate. A ratio of sample 2 (taken 18 d after TAI; d 18) to sample 1 (taken 3 d before TAI; d -3) was analyzed. The variable name "Oas1-s2/Oas1-s1" was used to define the d 18:d -3 ratio. Plasma progesterone concentrations were analyzed on d 18 by using a validated RIA (Kirby et al., 1997). The mean intraassay CV was 8.6%, and the interassay CV for a luteal phase control sample run in each assay was 3%. The cut-off value for nonpregnant diagnosis was <1.4 Oas1-s2/Oas1-s1 and (or) <2.0 ng/mL plasma progesterone.

A predetermined number of heifers were randomly assigned to the 21-d resynchronization program (21d_resynch; $n = 40$) or control ($n = 29$). The 21d_resynch heifers that were diagnosed as nonpregnant on d 18

were injected with PGF_{2α} (Lutalyse, i.m.) on d 19 and 48 h later received GnRH (100 µg of Cystorelin, i.m.) followed immediately by TAI. Heifers were observed for estrus twice daily until d 27, when pregnancy diagnosis by using an ultrasound was done for all heifers. Heifers that could not be definitively diagnosed as pregnant on d 27 were reexamined for pregnancy on d 34. The control heifers that were diagnosed not pregnant were resynchronized per veterinary recommendation according to their ovarian morphology at the time of the pregnancy exam. The typical program was that if a heifer had a CL, then she was treated with PGF_{2α} and inseminated 12 h after observed estrus. If no CL was present, then the heifer was enrolled in a CIDR-Ovsynch program (GnRH injection and CIDR insertion, 7-d interval, PGF_{2α} injection and CIDR removal, 2-d interval, GnRH injection and TAI). A final definitive pregnancy exam was done on d 60 to 62 after first and second insemination. No pregnancy losses occurred between the initial and final definitive pregnancy exams. Leukocyte Oas1-s2/Oas1-s1 and progesterone was analyzed for control heifers. This was done to generate additional data to estimate the accuracy of Oas1-s2/Oas1-s1 and progesterone tests, not for the purpose of pregnancy detection in control heifers. Binomial data (pregnancy per AI to first or second AI) were analyzed by Chi-squared test. Continuous dependent variables (e.g., leukocyte Oas1, plasma progesterone, interval to second insemination) were analyzed by using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). The statistical model included pregnancy status (pregnant or nonpregnant) and treatment.

Based on Oas1-s2/Oas1-s1 and progesterone, 21d_resynch heifers had a first TAI pregnancy per AI of 50% (20 pregnancies from 40 TAI). The 21d_resynch heifers diagnosed as nonpregnant at d 18 after TAI (n = 19) were treated for TAI on d 21. One 21d_resynch heifer was diagnosed as pregnant on d 18 but was observed in estrus on d 22 and re-inseminated (presumed to be nonpregnant). The control heifers had a first TAI pregnancy per AI of 51.7% (15 pregnancies out of 29 TAI). Some of the control heifers (n = 6) were re-inseminated after an observed estrus before pregnancy examination on d 27. The remaining control heifers that were diagnosed nonpregnant by transrectal ultrasonography on d 27 (n = 8) were resynchronized for AI per veterinary recommendation.

The Oas1-s2/Oas1-s1 ratio was approximately 5-fold greater for pregnant heifers compared with nonpregnant heifers (5.5 ± 0.5 and 1.2 ± 0.5 , respectively, $P < 0.001$). When individual components of the ratio were analyzed, heifers that were diagnosed pregnant by ultrasound on d 27 to first TAI had less Oas1 before first TAI (d -3) than heifers that were diagnosed not preg-

Figure 2. Amount of 2'-5' oligoadenylate synthetase 1 (Oas1) mRNA in peripheral blood leukocytes isolated 3 d before (d -3) fixed-time AI (TAI) and 18 d after TAI in dairy heifers that were subsequently diagnosed as pregnant (n = 35) or not pregnant (n = 34).

nant to first TAI (Figure 2; $P < 0.01$). On d 18, heifers diagnosed pregnant by ultrasound on d 27 to first TAI had more Oas1 expression than heifers diagnosed not pregnant to first TAI (Figure 2; $P < 0.001$). Heifers diagnosed pregnant to first TAI had greater plasma progesterone concentration on d 18 than heifers that were diagnosed not pregnant to first TAI (9.5 ± 0.7 and 5.9 ± 0.7 ng/mL, respectively; $P < 0.001$).

The pregnancy per AI to second (resynchronized) insemination was numerically less than first TAI pregnancy per AI (36.4 and 50.7% for second and first insemination, respectively; $P = 0.20$). No difference was detected for pregnancy per AI at second AI for 21d_resynch and control (36.8 and 35.7% for 21d_resynch and control, respectively). The interval between first and second AI was shorter for 21d_resynch than control (21.0 ± 0 d and 27.5 ± 2.1 d, respectively; $P < 0.02$). By design, on d 21 after AI, all (19/19) of the nonpregnant 21d_resynch heifers had been re-inseminated, whereas 35.7% (5/14) of the control heifers had been re-inseminated (Figure 3). The control heifers reached 100% re-insemination rate at d 51 after first TAI. The final percentage of pregnant heifers after 2 AI was similar for 21d_resynch and control (67.5 and 69.0%, respectively).

To our knowledge, this study represents the first attempt to perform pregnancy diagnosis and rapid resynchronization in dairy cattle within the first 21 d after AI. The method enables first insemination pregnancy diagnosis and resynchronization within the same estrous cycle. This approach has advantages given its timeliness (within 21 d) and the theoretical maturity of the CL and dominant follicle when the resynchronization is performed. The utility of this approach is well demonstrated by the survival plot for second insemina-

Figure 3. Survival plot for days to second AI for dairy heifers that were fixed-time AI (TAI) and then diagnosed not pregnant on d 18 and resynchronized for TAI on d 21 (21d_resynch) or were estrus detected and AI before d 27 and pregnancy tested on d 27 with re-insemination thereafter (control).

tion (Figure 3). All of the nonpregnant 21d_resynch heifers were re-inseminated on d 21, whereas the control heifers were re-inseminated over several weeks. Both approaches resulted in approximately 70% of the heifers becoming pregnant after 2 inseminations; however, the 21d_resynch group achieved this percentage 21 d after first TAI.

The 21d_resynch heifers could not be evaluated for false-negative diagnoses. This is because the heifers diagnosed as not pregnant were resynchronized for TAI before they could be examined for pregnancy by an alternative method. With this in mind, it is important to note that the first TAI pregnancy per AI was not different between the 21d_resynch and control groups. The possibility of an overwhelming number of false-negative diagnoses with Oas1-s2/Oas1-s1 and progesterone tests, therefore, is unlikely. We did have a false-positive diagnosis on d 18; this heifer returned to estrus on d 22 after first AI. Her Oas1-s2/Oas1-s1 ratio was 9.1 and her plasma progesterone concentration was 7.7 ng/mL. It is possible that she had an embryo that died after releasing interferon- τ and thus triggered an Oas1 response and extended the estrous cycle, but this could not be determined retrospectively.

We found elevated Oas1 before TAI (d -3) in heifers that failed to conceive to TAI (Figure 2). The possibility that Oas1 before insemination is predictive of TAI outcome should be considered cautiously, however, because we did not observe this relationship in our original work with heifers (Green et al., 2010). Future studies should address the relationship between prebreeding Oas1 concentrations and TAI outcome.

Three obvious caveats exist for this trial. First, it was conducted in heifers, whereas the typical target for resynchronization studies is the lactating cow. We ex-

amined the ISG response on d 18 in lactating cattle and found that the capacity to detect pregnancy with this method deteriorated as the cow aged (older lactating cows gave a weak signal on d 18, whereas first-parity cows and heifers had successively larger Oas1-s2/Oas1-s1 ratios if pregnant; Green et al., 2010). If techniques such as this are to be used in lactating cattle, then a suitable method for pregnancy diagnosis will need to be developed. If this method were developed, then the second aspect of the program (rapid resynchronization) in all likelihood would be successful. Indeed a preliminary study for lactating cows treated with rapid resynchronization on d 17 followed by TAI on d 19 achieved a 53% pregnancy per AI (n = 53 cows; Lucy et al., 2004). The second caveat to this study is that the second AI (resynchronized) pregnancy per AI was smaller than expected for both the 21d_resynch and control heifers (36.4%). We were surprised by this given the good fertility at first TAI (50.7%). It is possible that the relatively low pregnancy per AI at second insemination is simply an unfortunate outcome caused by relatively small groups (2 additional pregnancies per group would have increased the pregnancy per AI to $\geq 50\%$). It is also possible that additional fine-tuning of resynchronization is necessary. A third caveat relates to the fact that TAI was used for first insemination. The physiological response to the TAI program will have an effect on the return to estrus interval and could, therefore, affect the results of the resynchronization. For example, improperly synchronized heifers would be at an unexpected stage of the cycle on d 18 of this experiment. Leitman et al. (2009) demonstrated that the treatment sequence for CIDR and PGF_{2 α} used here was highly efficient for synchronizing estrus. We feel, therefore, that the possibility of incorrectly synchronized heifers was low but should be mentioned here.

The complexity of the procedure for pregnancy diagnosis described herein and the time required to do it precludes its practical on-farm application. Nonetheless, development of a similar method of pregnancy diagnosis that could be applied at the same time (≤ 18 d after AI) and on the farm would change the time when dairy cattle are resynchronized for second TAI. Producers could use TAI on cows or heifers that are not pregnant every 21 d by employing this method. The method does not depend on estrus detection. An important metric used by the dairy industry is the 21-d pregnancy rate (the percentage of eligible cows that become pregnant for a 21-d period). The 21d_resynch would yield a 21-d pregnancy rate that is identical to the TAI pregnancy per AI for a given herd. A typical US herd with a 21-d pregnancy rate of 15% and a TAI pregnancy per AI of 30%, therefore, could theoretically double their 21-d pregnancy rate by implementing this

method. Given the positive relationship between 21-d pregnancy rate and profitability of a dairy herd (De Vries, 2007), further research should be conducted to develop accurate and more practical methods of pregnancy diagnosis at ≤ 18 d after TAI.

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