Sync Programs and Ultrasound: Are We Getting in There too Early?

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Abstract

Early identification of non-pregnant dairy cows and heifers post-breeding can improve reproductive efficiency and pregnancy rate by decreasing the interval between artificial insemination (AI) services and increasing AI service rate. Thus, new technologies to identify non-pregnant dairy cows and heifers early after AI may play a key role in management strategies to improve reproductive efficiency and profitability on commercial dairy farms. Transrectal palpation is the oldest and most widely used method for early pregnancy diagnosis in dairy cattle[7]. However, a newer technology may someday replace transrectal palpation as the method of choice for pregnancy diagnosis in the dairy industry. Before this transition can occur, two events must transpire. First, a technology must be developed that exceeds transrectal palpation in one or more of the characteristics of the ideal early pregnancy test. Second and no less important, this new technology must be practically integrated into a systematic, on-farm reproductive management strategy and empirically demonstrated to exceed the status quo of the industry (i.e., transrectal palpation) in reproductive performance. Results from a recent study indicate that positive pregnancy outcomes diagnosed by transrectal ultrasonography conducted 26 days after timed AI (TAI) may be inflated due to pregnancy loss, compared to pregnancy outcomes conducted 33 days after TAI. Furthermore, fertility to TAI after re-sychronization of ovulation was greater when initiated 33 days after TAI compared to 26 days. These results suggest the counterintuitive notion that delaying pregnancy diagnosis from 26 to 33 days post-TAI may improve reproductive efficiency when using a hormonal protocol for timed AI to program non-pregnant cows for rebreeding. This is due to the high rate of pregnancy loss occurring in cows diagnosed pregnant at 26 versus 33 days post-TAI.

Résumé

L'identification précoce des vaches et taures laitières non-gestantes après l'insémination peut améliorer l'efficacité de la reproduction et le taux de gestation en réduisant l'intervalle entre les inséminations artificielles (IA) et en augmentant la fréquence des IA. De nouvelles technologies permettant l'identification précoce des vaches et taures laitières non-gestantes après l'IA pourrait donc jouer un rôle clef dans les stratégies de régie ayant pour but d'augmenter l'efficacité de la reproduction et la profitabilité dans les fermes laitières commerciales. La palpation transrectale est la plus ancienne méthode et celle encore la plus utilisée pour le diagnostic précoce de gestation chez les vaches laitières. Toutefois, une technologie plus nouvelle pourrait un jour remplacer la palpation transrectale comme méthode de choix pour le diagnostic de gestation dans l'industrie laitière. Deux choses devront prendre place avant que cette transition ne s'opère. En premier, la performance de la nouvelle méthode devra excéder celle de la palpation transrectale au niveau de l'une ou de plusieurs caractéristiques du test idéal de détection précoce de gestation. En second, et de façon tout aussi importante, cette nouvelle méthode devra s'intégrer pratiquement dans une stratégie systématique de régie de la reproduction à la ferme et démontrer empiriquement un niveau de performance au-delà de la norme actuelle de l'industrie (i.e. la palpation transrectale) en ce qui concerne la reproduction. Les résultats d'une étude récente indiquent que le nombre de conception diagnostiquée par l'ultrasonographie transrectale 26 jours après l'IA sur rendez-vous peut être surestimé à cause des pertes de grossesse par rapport à l'évaluation faite à 33 jours. De plus, la fertilité suite à l'IA sur rendez-vous avec resynchronisation de l'ovulation était plus élevée lorsqu'initiée 33 jours plutôt que 26 jours après l'IA sur rendez-vous. Ces résultats suggèrent contre intuitivement que de reporter le diagnostic de gestation de 26 jours à 33 jours suivant l'IA sur rendez-vous permettrait d'augmenter la performance de reproduction lorsqu'une gestion hormonale de l'IA sur rendez-vous est utilisée pour favoriser la reproduction chez les vaches non-gestantes. Ceci découle du taux élevé de perte de gestation chez les vaches diagnostiquées gestantes 26 jours plutôt que 33 jours suivant l'IA sur rendez-vous.

Return to Estrus as a Diagnostic Indicator of Pregnancy Status

Return to estrus from 18 to 24 days after AI is often considered by dairy farmers the easiest and least costly method for determining non-pregnancy in dairy cattle early post-breeding. This assumption, however, is being challenged by new research and long-recognized reproductive problems. First, estrous detection efficiency is estimated to be less than 50% on most dairy farms in the United States.[32] This is likely a result of the short duration of estrus behavior reported for lactating cows[9] and because cows display estrus behavior poorly when housed on concrete flooring,[38] a common housing situation for dairy cattle in many regions of the US and other countries. Second, estrous cycle duration varies widely among lactating dairy cows from the standard 21-day interval, and averaged around 24 days with a high degree of variability among lactating dairy cows.[31] This variability makes it difficult to detect return to estrus for groups of animals receiving AI on the same day. Finally, the high rate of pregnancy loss in dairy cows can increase the interval from insemination to return to estrus for cows that maintain a pregnancy, then lose that pregnancy later during gestation.[13] The rate of pregnancy loss is high during the gestation period when dairy cattle are submitted for pregnancy examinations using ultrasonography or rectal palpation and, therefore, is a key factor for understanding the implementation and implication of methods for early pregnancy diagnosis.

Pregnancy Loss in Dairy Cattle

Pregnancy loss contributes to reproductive inefficiency because fertility assessed at any point during pregnancy is a function of both conception rate and pregnancy loss.[12] Since the widespread implementation of transrectal ultrasonography for reproductive research in cattle,[18] several studies have reported rates of pregnancy loss during early gestation under field conditions. Table 1 summarizes reported rates of pregnancy loss in lactating dairy cows from an initial pregnancy diagnosis conducted 27 to 30 days post breeding to a subsequent pregnancy reassessment 14 to 42 days later. Taken together, average pregnancy loss reported in these studies exceeded 15%. Vasconcelos *et al* [40] characterized pregnancy loss at various stages of gestation using transrectal ultrasonography. They reported pregnancy losses of 11% from 28 to 42 days, 6% from 42 to 56 days and 2% from 56 to 98 days post AI, suggesting the rate of loss is greater in early gestation, then decreases as gestation proceeds.

Early pregnancy diagnosis can improve reproductive performance by decreasing the interval between successive AI services and coupling a non-pregnancy diagnosis with an aggressive strategy to rapidly re-breed these animals.[12] Conversely, it has long been accepted that pregnancy status should be determined in dairy cattle as soon as possible after insemination, but without having the diagnosis confounded by subsequent pregnancy loss.[20,37] Pregnancy loss diminishes the benefit of early pregnancy diagnosis in two ways. First, because of the high rate of pregnancy loss that occurs around the time of gestation when most direct and indirect pregnancy tests are performed (Table 1), the magnitude of pregnancy loss detected is greater the earlier post-breeding that a positive diagnosis is made. Thus, the earlier that pregnancy is diagnosed post-breeding, the fewer non-pregnant cows are identified to which a management strategy must be implemented to rebreed them. Second and more important, cows diagnosed pregnant earlier post-breeding have a greater risk for pregnancy loss, compared to cows diagnosed later post-breeding. If left unidentified, cows diagnosed pregnant early post-breeding that subsequently lose that pregnancy reduce reproductive efficiency by extending the interval from calving to the conception that results in a full-term pregnancy.

Table 1. Pregnancy loss in lactating dairy cows occurring from first early pregnancy diagnosis conducted from 27 to 30 days post breeding to a pregnancy recheck conducted 14 to 42 days later.

Number of pregnancies evaluated	Days of gestation at diagnosis		Loss interval, d	Pregnancy loss, %	Reference
	First	Second			
256	28	38-58	~ 20	28.0	Cartmill *et al*[5]
195	28	42	14	17.9	Chebel *et al*[6]
89	28	56	28	13.5	Fricke *et al*[14]
209	26	68	42	27.8	Fricke *et al*[13]
77	33	68	35	11.7	Fricke *et al*[13]
139	27	45	18	20.7	Moreira *et al*[21]
172	28	45	17	9.3	Santos *et al*[30]
372	31	45	14	11.4	Santos *et al*[28]
215	27	41	14	9.9	Santos *et al*[29]
705	28	42	14	3.2	Silke *et al*[33]
347	33	61	28	6.6	Sterry *et al*[34]

To compensate for pregnancy loss, cows diagnosed pregnant early post-breeding must undergo one or more subsequent pregnancy reconfirmations to identify and re-breed cows that experience pregnancy loss. Thus, dairy mangers who have implemented early pregnancy diagnoses must consider the timing and frequency of subsequent pregnancy examinations to maintain the reproductive performance of the herd. Problems caused by pregnancy loss apply to all currently available methods for assessing pregnancy status early post-breeding, and may relegate pregnancy testing before 30 to 40 days post-breeding as an untenable management strategy unless pregnancy diagnoses can be made continually on a daily basis or at each milking until the rate of pregnancy loss decreases, or until the underlying causes of pregnancy loss are understood and mitigated.

Attributes of the Ideal Pregnancy Test

For successful integration into a reproductive management system, an ideal early pregnancy test for dairy cattle would be 1) sensitive (i.e., correctly identify pregnant animals), 2) specific (i.e., correctly identify non-pregnant animals), 3) inexpensive, 4) simple to conduct under field conditions, and 5) able to determine pregnancy status at the time the test is performed. Most currently available methods for pregnancy diagnosis exhibit one or more of these attributes, but none currently available or under development exhibit all of them. A final attribute of an ideal test would be the ability to determine pregnancy status without the need to physically handle the animal to administer the test. Such a test may overcome the inherent limitations of current tests caused by pregnancy loss and may make pregnancy diagnosis before 30 to 40 days postpartum in dairy cattle an economically viable reproductive management strategy. Although rectal palpation and transrectal ultrasonography both require animal handling to administer the test, future strategies and technologies for early pregnancy diagnosis may someday realize this goal.

Transrectal Palpation

Transrectal palpation of the uterus for pregnancy diagnosis in cattle was first described in the 1800's[7] and is the oldest and most widely used method for early pregnancy diagnosis in dairy cattle today. Palpation technique can vary among practitioners. Transrectal palpation of the amniotic vesicle as an aid in determining pregnancy status in dairy cattle was described by Wisnicky and Cassida,[42] whereas slipping of the chorioallantoic membranes between the palpator's thumb and forefinger beginning on about day 30 of gestation was described by Zemjanis.[43] Veterinary schools across the US and in other countries continue to train their students in the art of transrectal palpation for diagnosis of pregnancy in dairy cattle.

Because pregnancy in cattle can be terminated by manual rupture of the amnionic vesicle,[2] many studies have investigated the extent of iatrogenic pregnancy loss induced by transrectal palpation. Several studies have suggested that examining pregnant cows early in gestation by transrectal palpation increases the risk of iatrogenic pregnancy loss,[1,11,24,39,41] whereas other studies have suggested that cows submitted for transrectal palpation earlier during gestation had a decreased risk for abortion or that palpation had no effect on subsequent embryonic losses.[35,37] Although controversy still exists regarding the extent of iatrogenic pregnancy loss induced by transrectal palpation, other factors have a greater influence on calving rates than pregnancy examination by transrectal palpation.[36] Furthermore, because the risk of pregnancy loss is high during the period of gestation when cows are diagnosed pregnant by transrectal palpation (Table 1), and because most cows within a herd are submitted for pregnancy examination, it is impossible for dairy producers and veterinarians to distinguish between iatrogenic losses occurring due to transrectal palpation and spontaneous losses that would normally have occurred in these cows.

Because of its widespread use and the number of bovine practitioners trained to perform the procedure, transrectal palpation will likely remain a mainstay for pregnancy diagnosis in dairy cattle until a newer method for pregnancy diagnosis is developed that exceeds the technique in one or more of the attributes of the ideal pregnancy test. Furthermore, because of its widespread use, high accuracy, and relatively low cost per animal, transrectal palpation is the industry standard that newer methods for pregnancy diagnosis in dairy cattle must displace as the method of choice for pregnancy diagnosis.

B-Mode Ultrasonography

Applications of and detailed methods for performing transrectal ultrasonography for reproductive research have been reviewed and described in detail.[17,18] Most veterinary students continue to be taught that ultrasound is a secondary technology for bovine reproductive work; however, the information-gathering capabilities of ultrasonic imaging far exceed those of transrectal palpation.[16] Although early pregnancy diagnosis is among the most practical application for reproductive management using transrectal ultrasonography, additional information gathered using the technology that may be useful for reproductive management include evaluation of ovarian structures, identification of cows carrying twin fetuses and determination of fetal sex.[12] A fetal heartbeat can be visualized at around 21 days of gestation under controlled experimental conditions using a high-quality scanner and transducer,[8] and represents the definitive characteristic for positive confirmation of a viable pregnancy using transrectal ultrasonography. Although the rate of pregnancy loss is significant in studies using ultrasound to assess the rate of loss (Table 1), the technique itself has not been implicated as a direct cause of pregnancy loss in cattle.[3,4] Ultrasound is a less-invasive technique for early pregnancy diagnosis than is transrectal palpation[24,39] and may minimize incidence of palpation-induced abortions.

Under most on-farm conditions, pregnancy diagnosis can be rapidly and accurately diagnosed using ultrasound as early as 26 days post-AI.[10,19] When conducted between 21 and 25 days post breeding, sensitivity and specificity of pregnancy diagnosis using ultrasound was 44.8% and 82.3%, respectively, but increased to 97.7% and 87.7%, respectively, when conducted between 26 and 33 d post AI.[25] Sensitivity and specificity of pregnancy diagnosis in lactating dairy cows based on ultrasonographic detection of uterine fluid, as well as embryonic membranes, from 28 to 35 days after AI was 96% and 97%, respectively.[22] Pregnancy diagnosis in dairy heifers based on the presence of intraluminal uterine fluid before day 16, however, is unreliable because small amounts of fluid are present in non-inseminated heifers as early as 10 days after estrus.[19] For lactating dairy cows, ultrasonographic detection of uterine fluid as well as embryonic membranes from 28 to 35 days after AI was an accurate estimation of the presence of an embryo at the time of observation.[22] Although ultrasound conducted at ≥ 45 days post-breeding did not increase accuracy of pregnancy diagnosis for an experienced palpator, it may improve diagnostic accuracy of a less experienced one.[15]

As a pregnancy diagnosis method, transrectal ultrasonography is accurate, and the outcome of the test is known immediately at the time the test is conducted. Veterinary-grade ultrasound machines equipped with one rectal transducer are expensive ($8,000 to $16,000).[12] The cost of this technology may limit its practical implementation. Although dairy producers can purchase an ultrasound scanner and conduct pregnancy examinations on their own cows, they generally lack the knowledge, training and experience required to accurately perform pregnancy examinations.[12] Transrectal ultrasonography is slowly being incorporated into reproductive management schemes in dairies primarily by bovine practitioners who have adopted this technology. The extent to which transrectal ultrasonography will displace transrectal palpation as the primary direct method for pregnancy diagnosis in dairy cattle remains to be seen. Because many experienced bovine practitioners can accurately diagnose pregnancy as early as 35 days post breeding using transrectal palpation, pregnancy examination using transrectal ultrasonography at 26 to 28 days post-breeding only reduces the interval from insemination to pregnancy diagnosis by seven to nine days. The rate of pregnancy loss and the efficacy of strategies to re-breed cows at various stages post-breeding also play a role in determining the advantages and disadvantages on the timing of pregnancy diagnosis and resynchronization.[13]

On-Farm Implementation of Early Non-pregnancy Diagnosis

Synergies between new reproductive management technologies hold the key to maximizing reproductive efficiency on dairy farms. However, reproductive management protocols that allow for synchronization of ovulation and subsequent identification and resynchronization of non-pregnant cows must be practical to implement within the day-to-day operation of a dairy farm, or the protocol will fail due to lack of compliance.[13] This is especially true for larger farms that must schedule and administer artificial inseminations, hormone injections and pregnancy tests for a large number of animals on a daily or weekly basis. Identification of non-pregnant cows early post-breeding can only improve reproductive efficiency when coupled with a management strategy to rapidly submit non-pregnant cows for a subsequent AI service. Thus, any method for early pregnancy diagnosis must be integrated as a component of the overall reproductive management strategy in place on the farm. The various component technologies of the reproductive management system will, in turn, determine the timing of events as they occur on a daily or weekly basis. As stated previously, it has long been accepted that pregnancy status should be determined in dairy cattle as soon as possible after insemination, but without having the diagnosis confounded by subsequent pregnancy loss.[20,35] New research on the practical implementation of early pregnancy diagnosis using transrectal ultrasonography into a systematic synchronization and resynchronization system has confirmed this notion, and illustrated the pitfalls and limitations of early pregnancy diagnosis.[13]

Field Trial: Integrating Systematic Synchronization with Transrectal Ultrasonography

Two recently-adopted technologies for reproductive management of dairy cattle include hormonal protocols such as Ovsynch[26,27] and Presynch/Ovsynch,[21,23] and use of transrectal ultrasonography for early identification of non-pregnant cows.[12] We conducted a field trial to compare three intervals from first TAI to resynchronization of ovulation on a dairy, incorporating transrectal ultrasonography as a method for early pregnancy diagnosis.[13] The objective was to compare conception rate to first TAI service after a modified Presynch protocol with conception rates after resynchronization of ovulation, using Ovsynch at three intervals post-TAI (Resynch), coupled with pregnancy diagnosis using transrectal ultrasonography. Lactating dairy cows on a commercial dairy farm were enrolled into this study on a weekly basis.

All cows received a modified Presynch protocol to receive first postpartum TAI as follows: 25 mg prostaglandin F_2 ($PGF_{2\alpha}$; day 32 ± 3; day 46 ± 3); 50 µg gonadotropin-releasing hormone (GnRH) (day 60 ± 3); 25 mg $PGF_{2\alpha}$ (day 67 ± 3) and 50 µg GnRH (day 69 ± 3) postpartum.[23] All cows received TAI immediately after the second GnRH injection of the Presynch protocol (day 0) as per a Cosynch TAI schedule. At first TAI, cows were randomly assigned to each of three treatment groups for resynchronization of ovulation (Resynch) using Ovsynch [(50 µg GnRH [day -9]; 25 mg $PGF_{2\alpha}$ [day -2] and 50 µg GnRH + TAI [day -0]) to induce a second TAI for cows failing to conceive to first TAI service. All cows (n=235) in the first group (D19) received a GnRH injection on day 19 post-TAI and continued the Ovsynch protocol if diagnosed non-pregnant using transrectal ultrasound on day 26 post-TAI. Cows (n=240) in the second (D26) and cows (n=236) in the third (D33) groups initiated the Ovsynch protocol if diagnosed nonpregnant using transrectal ultrasound on day 26 post-TAI or day 33 post-TAI, respectively. Submission of cows for first postpartum TAI service was scheduled so that the first four injections of the Presynch plus Ovsynch protocol occurred on Tuesdays, followed by the second GnRH injection and TAI occurring on Thursdays (Table 2). Initiation times for Resynch for each of the three treatment groups in this study were chosen to occur on Tuesdays so that injection schedules would remain consistent for all cows assigned to weekly breeding groups at any given time. To adhere to the Tuesday/Thursday schedule, all pregnancy examinations were conducted on Tuesdays. To fit the reproductive management system, the first pregnancy examination using transrectal ultrasound was conducted 26 days after TAI for the D19 and D26 cows, and 33 days after TAI for the D33 cows (Figure 1).

Implicit to the experimental design, first assessment of pregnancy status was not conducted at the same interval after the Ovsynch TAI among the three treatment groups. Pregnancy status after the Ovsynch TAI was first assessed 26 days after TAI for cows in the D19 and D26 groups, whereas pregnancy status was assessed 33 days post-Ovsynch TAI for cows in the D33 group. Overall fertility to Ovsynch was 40% and was greater for D19 and D26 cows than for D33 cows (Table 3). This difference is likely due to a greater period in which pregnancy loss can occur in the D33 cows, due to the increased interval from TAI to pregnancy diagnosis (26 vs. 33 days). When pregnancy status was reassessed for all treatment groups at 68 days after Ovsynch TAI, overall pregnancy rate per AI (PR/AI) to Ovsynch was 31% and did not differ among treatments (Table 3). Thus, differences in PR/AI at the first pregnancy exam, and pregnancy losses between the first and second pregnancy

Table 2. One possible schedule for administering hormone injections, timed artificial insemination, and pregnancy diagnosis using transrectal ultrasonography for the Presynch/Ovsynch protocol for first TAI and Resynchronization for second TAI based on the Day 33 Resynch treatment reported by Fricke *et al.*[13] Note that all hormone injections, timed artificial inseminations and pregnancy examinations are restricted to two days of the week.

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
week 1		PGF				
week 2						
week 3		PGF				
week 4						
week 5		GnRH				
week 6		PGF		GnRH+TAI		
week 7						
week 8						
week 9						
week 10						
week 11		GnRH				
week 12		PG+PGF		GnRH+TAI		

PGF = prostaglandin $F_{2\alpha}$, GnRH = gonadotropin-releasing hormone, TAI = timed artificial insemination, PG = pregnancy diagnosis using transrectal ultrasonography.

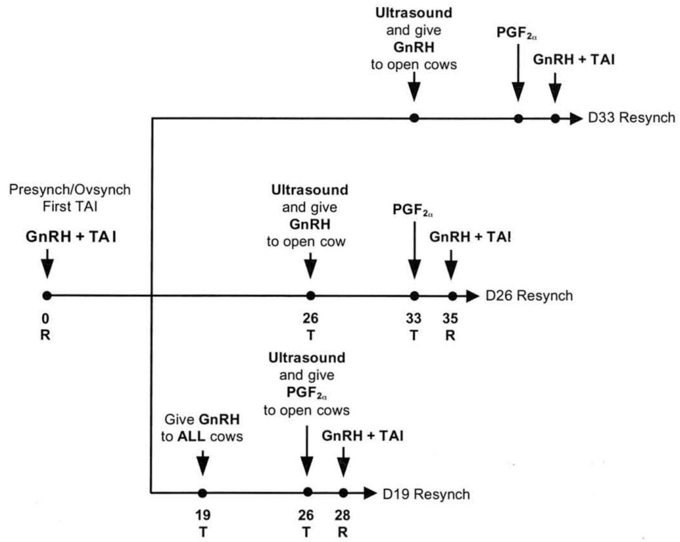

Figure 1. Diagram of resynchronization treatment groups from Fricke *et al.*[13] Pregnancy rate per artificial insemination (PR/AI) and pregnancy loss from first pregnancy evaluation to a second reconfirmation were evaluated to determine the best method for integration of early pregnancy diagnosis using transrectal ultrasonography.

exams, among treatment groups likely represent an artifact of time of assessment of pregnancy status after TAI, inherent to the experimental design rather than to treatment differences. Overall PR/AI to Resynch was 32% and was greater for D26 and D33 cows than for D19 cows (Table 4).

The Challenges for Early Pregnancy Diagnosis

Data from Tables 3 and 4 illustrate the limitations of integrating early pregnancy diagnosis into a reproductive management program. First, the system with the most aggressive early non-pregnancy diagnosis and resynchronzation schedule (i.e., the D19 treatment) was not a viable management strategy based on the poor fertility after the Resynch TAI (Table 4), probably due to follicular and luteal dynamics at the stage post-breeding that the synchronization protocol was initiated. Furthermore, these results suggest the counterintuitive notion that delaying pregnancy diagnosis from 26 to 33 days post-TAI may improve reproductive efficiency when using a hormonal protocol for timed AI to program non-pregnant cows for rebreeding, due to the high rate of pregnancy loss occurring in cows diagnosed pregnant at 26 vs. 33 days post-TAI (Table 3).

Conclusion

Although coupling a nonpregnancy diagnosis with a management decision to quickly reinitiate AI service may improve reproductive efficiency by decreasing the interval between AI services, early pregnancy loss and the effectiveness of hormonal ovulation and estrus control protocols initiated at certain physiologic stages post-breeding may limit the effectiveness of many methods for early pregnancy diagnosis currently under development, especially when compared to transrectal palpation. These limitations make the benefits of many currently available methods for early pregnancy diagnosis questionable, and require that all animals diagnosed pregnant early after insemination be scheduled for rechecks at later times during gestation to identify animals experiencing pregnancy loss. It remains to be seen whether a new test will replace transrectal palpation as the primary method used for pregnancy diagnosis in dairy cattle.

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Table 3. Pregnancy rate per artificial insemination (PR/AI) and pregnancy loss after timed artificial insemination (TAI) to Ovsynch (Adapted from Fricke *et al*[13]).

Item	Treatment group D19	D26	D33	Overall
Interval from Ovsynch TAI to 1st pregnancy exam (d)	26	26	33	-
PR/AI at 1st pregnancy exam, %	46[a]	42[a]	33[b]	40
(no./no.)	(108/235)	(101/240)	(77/236)	(286/711)
Interval from Ovsynch TAI to 2nd pregnancy exam (d)	68	68	68	-
PR/AI at 2nd pregnancy exam, %	33	30	29	31
(no./no.)	(78/235)	(73/240)	(68/236)	(219/711)
Interval between pregnancy exams (d)	42	42	35	-
Pregnancy loss, %	28[a]	28[a]	12[b]	23
(no./no.)	(30/108)	(28/101)	(9/77)	(67/286)

[a,b]Within a row, percentages with different superscripts differ ($P < 0.01$) among treatment groups.

Table 4. Pregnancy rate per artificial insemination (PR/AI) after timed artificial insemination (TAI) to Resynch beginning 19, 26, or 33 days after first TAI (Adapted from Fricke *et al*[13]).

Item	Treatment group D19	D26	D33	Overall
Mean (± SEM) interval (d) from Resynch TAI to pregnancy exam (range)	27.1 ± 0.4 (26 to 54)	26.6 ± 0.2 (26 to 40)	33.7 ± 0.4 (26 to 75)	-
PR/AI, %	23[a]	34[b]	38[b]	32
(no./no.)	(28/120)	(41/121)	(54/143)	(123/384)

[a,b]Within a row, percentages with different superscripts differ ($P < 0.01$) among treatment groups.

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