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Resynchronization of ovulation and timed insemination in lactating dairy cows I: use of the Ovsynch and Heatsynch protocols after non-pregnancy diagnosis by ultrasonography

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Abstract

The objective was to compare pregnancy rates and pregnancy losses in lactating dairy cows that were diagnosed not pregnant and re-inseminated following either the Ovsynch® or Heatsynch protocols. Also evaluated were the effects of stages of the estrous cycle, ovarian cysts and anestrus on pregnancy rates for both treatments. Non-pregnant cows ($n = 332$) as determined by ultrasonography on day 27 post-AI (study day 0) were divided into two groups. Cows in the Ovsynch group ($n = 166$) received GnRH on day 0, $PGF_{2\alpha}$ on day 7, GnRH on day 9, and timed AI (TAI) 16 h later (day 10). Cows in the Heatsynch group ($n = 166$) received GnRH on day 0, $PGF_{2\alpha}$ on day 7, estradiol cypionate (ECP) on day 8, and TAI 48 h later (day 10). Cows detected in estrus on days 8 and 9 were inseminated and included in the study. On day 0, cows were classified according to different stages of the estrous cycle, or presence of ovarian cysts or anestrus. Pregnancy rates were evaluated 27, 45 and

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90 days after resynchronized AI. Overall, there was no difference in pregnancy rates on days 27, 45 and 90 between cows in the Ovsynch (25.2, 17.5, and 13.9%) and Heatsynch (25.8, 19.9, and 16.1%) groups. There was no difference in pregnancy losses from days 27 to 45 and days 45 to 90 for cows in the Ovsynch (25.0 and 17.9%) and Heatsynch (14.7 and 10.3%) groups. However, pregnancy rates were increased when cows in metestrus were subjected to the Heatsynch protocol and cows with ovarian cysts were subjected to the Ovsynch protocol.

Keywords: Resynchronization; Timed-AI; Dairy cows; Estrous cycle

1. Introduction

Reproductive efficiency in dairy herds increases by inseminating all the cows shortly after the end of the voluntary waiting period, obtaining high pregnancy rate to first service, enhancing embryonic and fetal survival, and detecting and re-inseminating non-pregnant cows. Ultrasonography enables early pregnancy diagnosis and detection of non-pregnant cows [1,2], which can be subjected to resynchronization of ovulation and timed AI (TAI) to minimize the problem of low estrus detection [3].

Protocols for synchronization of ovulation and TAI, such as Ovsynch (GnRH on day 0, $PGF_{2\alpha}$ on day 7, GnRH on day 9 and TAI 16 h later; [4,5]) and Heatsynch (GnRH on day 0, $PGF_{2\alpha}$ on day 7, ECP on day 8, and TAI on day 10; [6]), insure that all cows are inseminated and generate acceptable pregnancy rates in lactating dairy cows. Since the stage of the estrous cycle is known to affect the response to these protocols [7,8], a presynch program was developed that allows initiation of Ovsynch in the early- to mid-luteal phase of the estrous cycle, improving pregnancy rate to first service in cyclic cows [9].

Protocols for resynchronization of estrus that considered the stage of the estrous cycle have been applied after detection of non-pregnant cows by per rectum palpation of the uterus and assignment of protocols based on the presence or absence of a CL [10]. Another approach for resynchronization after non-pregnancy diagnosis by ultrasonography was either to initiate the Ovsynch protocol 7 days before ultrasonography [11–13] or to take advantage of a natural resynchronization after previous service with application of shortened protocols that used $PGF_{2\alpha}$ to induce luteolysis and then GnRH [14] or ECP [15,16] to induce ovulation. An alternative is to consider the stage of the estrous cycle at the time of initiation of the resynchronization protocol by evaluating ovarian structures and uterine characteristics at the time of a non-pregnancy diagnosis. Determination of the stage of the estrous cycle can be done using per rectum examination [17] and ultrasonography [18,19] of the genital tract.

The objective of this study was to compare pregnancy rates on days 27, 45 and 90 and pregnancy losses in lactating dairy cows that were diagnosed not pregnant at ultrasonography on day 27 after a previous AI and re-inseminated following either Ovsynch or Heatsynch protocols. In addition, we evaluated the effect of stages of the estrous cycle, or presence of ovarian cysts or anestrus at the time of initiation of these protocols on pregnancy rates for both treatments.

2. Materials and methods

2.1. Study population

The study was conducted from August to December 2001 in a large commercial dairy with 3200 milking cows. The herd is in north central Florida and is divided into 14 lots according to levels of production and stage of lactation and cows from all lots were included in the study. Cows were housed in free stall barns and dry lots and fed a total mixed ration (TMR) thrice daily. The TMR was formulated to meet or exceed requirements for lactating cows [20]. Cows were milked three times a day and had a rolling herd average for milk production of 10,700 kg. Beginning at 60 days postpartum, cows received a bST treatment (Posilac®, 500 mg sometribove zinc, subcutaneously; Monsanto, St Louis, MO, USA) every 14 days during remainder of lactation. Reproductive management consisted of a voluntary waiting period of 75 days that incorporated a Presynch–Ovsynch program [9] for first service, and estrus detection using visual observation and a computerized pedometer system (Afimilk®, S.A.E. Afikim, Kibbutz Afikim, Israel) for subsequent services. The study included 332 lactating dairy cows detected non-pregnant by ultrasonography at day 27 (study day 0) after AI. Cows with reproductive abnormalities (i.e., metritis, pyometra, uterine or ovarian adhesions) were not included in the study.

2.2. Study design

On day 0, non-pregnant cows ($n = 332$) were divided randomly biweekly into two groups. Cows in the Ovsynch group ($n = 166$) received 100 μg im of GnRH (2 mL of Cystorelin®; Merial Ltd., Iselin, NJ, USA) on day 0, 25 mg im of $PGF_{2\alpha}$ (5 mL of Lutalyse® sterile solution; Pfizer Animal Health, New York, USA) on day 7, 100 μg im of GnRH on day 9, and TAI 16 h later (day 10). Cows in the Heatsynch group ($n = 166$) received 100 μg im of GnRH on day 0, 25 mg im of $PGF_{2\alpha}$ on day 7, 1 mg im of ECP (0.5 mL of ECP® sterile solution; Pfizer Animal Health) on day 8, and TAI 48 h later (day 10). Cows detected in estrus on days 8 or 9 in both groups received either GnRH or ECP according to the protocol and were inseminated and included in the study. On day 0, information for parity, time of the year (season), days in milk (DIM), and inseminator were recorded.

2.3. Determination of stages of the estrous cycle, ovarian cysts or anestrus

On day 0, cows were classified according to different stages of the estrous cycle, or presence of ovarian cysts or anestrus combining per rectum examination [17] and ultrasonography of the genital tract [18,19] to determine ovarian structures and uterine characteristics (Table 1). Detection of a functional CL included morphology at palpation (i.e., line of demarcation and distortion in the shape of the ovary) and visualization at ultrasonography. A valid criticism for diagnosis of a functional CL is that plasma progesterone was not determined; therefore, there could be a period of a few hours after $PGF_{2\alpha}$ release that a CL can be palpated and observed at ultrasonography, but plasma progesterone concentration would be less than 1 ng/mL. At 24 and 48 h after $PGF_{2\alpha}$

Table 1
Criteria for determination of the stage of the estrous cycle, or presence of ovarian cysts or anestrus based on ultrasonography and per rectum palpation of the genital tract (adapted from 17,18, and 19)

Stage	Clinical findings	
	Ovaries	Uterus
Diestrus	Functional CL, follicle $>$ 10 mm	Slight tonus
Metestrus	Corpus hemorrhagicum, follicle $<$ 10 mm	Edema and moderate tonus
Proestrus/estrus	Follicle $\sim$ 18 mm, regressing CL	High tonus
Ovarian cysts	Multiple follicles $\sim$ 18 mm, absence of CL	Flaccid
Anestrus	Follicle $<$ 18 mm	Flaccid

exposure, the CL may be observed at ultrasonography but CL regression is evident at palpation per rectum (i.e., hard CL and uterine tone). Detection of follicles was based on ultrasonography, uterine tonicity using per rectum palpation and uterine edema using both ultrasonography and per rectum palpation. Per rectum palpation and ultrasonography of the genital tract have been previously used for the diagnosis of ovarian cysts [21]. In the present study, the diagnosis of ovarian cysts was based on three clinical findings: (1) multiple follicles approximately 18 mm in diameter; (2) absence of a CL; and (3) lack of uterine tonicity. Previous studies have defined ovarian cysts as follicles greater than 15–20 mm in diameter [17,22–28]. Collectively, these studies indicate that a large follicle (e.g., 25 mm) can not be used as the sole criterion for cysts since multiple follicles reach ovulatory size, indicating enough gonadotropin to support follicular development, but these follicles fail to ovulate due to lack of GnRH/LH surge. The absence of uterine tonicity is the most important clinical finding that can be used to make a diagnosis on a single examination since it indicates that regression of the CL has occurred, the cow has failed to ovulate, and the follicles are continuing to grow in the absence of high progesterone concentrations [17,22].

2.4. Pregnancy diagnosis to resynchronized AI

Pregnancy diagnosis was conducted 27 ($\pm$1) days after the resynchronized AI using a linear array with an Aloka 500V ultrasound scanner with 5-MHz linear-array, transrectal transducer machine (Wallingford, CT, USA). Cows were classified as: (1) pregnant: embryo proper with heartbeats and surrounded by a fluid-filled cavity representing the allantoic cavity in the uterine lumen [1,2]; (2) questionable pregnant: detection of fluid in the uterine lumen and a CL, but no embryo observed; or (3) not pregnant: cows that were either AI at detected estrus following the resynchronized AI and prior to ultrasonography or diagnosed not pregnant at ultrasonography. On days 45 and 90, pregnancy status was determined by per rectum examination of the genital tract based on previously described criteria [17]. On day 27 + 1 after the resynchronized TAI, 259 (127 in Ovsynch and 132 in Heatsynch) cows were presented for pregnancy diagnosis and 69 cows were not presented. On day 45, 332 cows (166 in Ovsynch and 166 in Heatsynch) were evaluated for pregnancy diagnosis. On day 90, five cows that were pregnant on day 45 were culled from the herd and 327 cows (165 in Ovsynch and 162 in Heatsynch) were evaluated for pregnancy. Among cows evaluated at day 45 and day 90 for pregnancy, cows diagnosed non-pregnant at day 27 and day 45 were not palpated subsequently at day 45 and day 90, respectively. Cows classified as questionable pregnant on day 27 were re-examined at day 45.

2.5. Statistical analysis

Baseline comparisons for parity (1, 2, 3+), season (August to September and October to December), DIM (quartiles), inseminator (A, B, C, D, E, and F), stages of the estrous cycle (diestrus, metestrus, and proestrus), ovarian cysts or anestrus were carried out to establish comparability among groups using a Chi-square test (PROC FREQ, SAS system). Pregnancy rates on days 27, 45 and 90 and pregnancy losses between days 27 and 45 and 45 and 90 for both groups were compared using Chi-square. In addition, pregnancy rates on days 27, 45 and 90 were compared using a multivariate analysis to adjust for differences in baseline data and to evaluate the interactions between groups and stages of the estrous cycle, or presence of ovarian cysts or anestrus on pregnancy rate. The model included group, stage (i.e., stages of the estrous cycle, or presence of ovarian cysts or anestrus), parity, season, DIM, inseminator and their interactions, and they remained in the model at $P < 0.15$ using the backward elimination method of the logistic regression procedure in PROC GENMOD, SAS [29]. Treatment differences with $P \leq 0.05$ were considered significant, whereas tendencies were considered when $P > 0.05$ and $P \leq 0.15$.

3. Results

There was no difference in the distributions of cows by parity ($P = 0.56$), season ($P = 0.91$) or DIM ($P = 0.83$). Distributions of cows among inseminators were different ($P < 0.01$) between groups. The distribution of cows in different stages of the estrous cycle, with ovarian cysts or anestrus on day 0 ($n = 332$) was: diestrus 46.1%; metestrus 14.8%; proestrus 22.0%; ovarian cysts 14.4%; and anestrus 2.7%. The distributions of cows by stage of the estrous cycle, or presence of ovarian cysts or anestrus were not different ($P = 0.85$) between groups.

The numbers of cows inseminated at estrus on days 8 to 9 were lower ($P < 0.01$) in the Ovsynch group [15.1%, 25/166 (10/81 diestrus, 1/22 metestrus, 8/35 proestrus, 6/23 ovarian cyst and 0/5 anestrus)] than in the Heatsynch group [36.1%, 60/166 (25/72 diestrus, 11/27 metestrus, 12/38 proestrus, 11/25 ovarian cyst and 1/4 anestrus)].

No differences were detected between treatment groups for pregnancy rates on days 27, 45 and 90 or pregnancy losses between days 27 and 45, or between days 45 and 90 (Table 2). In the multivariate analysis for pregnancy rates, there was an interaction ($P < 0.01$) between treatment group and stages of the estrous cycle, presence of ovarian cysts or anestrus. Therefore, pregnancy rates were evaluated for both protocols in cows at different stages of the estrous cycle, and with ovarian cysts using logistic regression (group, stage, ovarian cysts, parity, season, DIM, inseminator and their interactions were included in the model). Cows in anestrus were not considered in the statistical analysis due to the low number of cows in this category. Cows in diestrus subjected to the Ovsynch protocol were selected as the referent group since previous information demonstrated higher pregnancy rate when Ovsynch was initiated during early or mid-luteal phases of diestrus [9].

Table 2
Pregnancy rates on days 27, 45, and 90 and pregnancy losses between days 27 and 45 and days 45 and 90 for cows in the Ovsynch and Heatsynch groups

Variable	Group		*P* value
	Ovsynch % (N)	Heatsynch % (N)	
Pregnancy rate			
Day 27	25.2 (127)	25.8 (132)	0.91
Day 45	17.5 (166)	19.9 (166)	0.57
Day 90	13.9 (165)	16.1 (162)	0.59
Pregnancy losses			
Days 27–45	25.0 (32)	14.7 (34)	0.29
Days 45–90	17.9 (28)	10.3 (29)	0.41

The risk of non-pregnancy on day 27 was higher for cows with ovarian cysts subjected to the Heatsynch protocol compared to cows in diestrus subjected to Ovsynch, and tended to decrease for cows in metestrus subjected to Heatsynch compared to cows in diestrus subjected to Ovsynch (Table 3).

The risk of non-pregnancy on day 45 tended to increase in cows with ovarian cysts subjected to Heatsynch compared to cows in diestrus subjected to Ovsynch, and decreased for cows in metestrus subjected to Heatsynch compared to cows in diestrus subjected to Ovsynch (Table 4).

The risk of non-pregnancy on day 90 tended to increase for cows with ovarian cysts subjected to Heatsynch, increased for cows in metestrus subjected to Ovsynch compared to cows in diestrus subjected to Ovsynch, and tended to decrease for cows in metestrus subjected to Heatsynch compared to cows in diestrus subjected to Ovsynch (Table 5).

4. Discussion

The importance of reducing the interval between first and second AI has stimulated the development of different strategies to resynchronize return to estrus [12–16,30,31].

Table 3
Pregnancy rates on day 27, odds ratio (OR), 95% confidence interval (CI) and levels of significance for the risk of non-pregnancy for cows in the Ovsynch and Heatsynch groups and at different stages of the estrous cycle, or presence of ovarian cysts

Variable		Pregnancy rate % (N)	OR	95% CI	*P* value
Group	Ovarian classification				
Ovsynch	Diestrus	30.2 (63)	Referent	Referent	–
Heatsynch	Diestrus	24.1 (58)	1.3	0.6–3.0	0.45
Ovsynch	Metestrus	15.4 (13)	2.4	0.5–11.8	0.29
Heatsynch	Metestrus	52.2 (23)	0.4	0.1–1.0	0.06
Ovsynch	Proestrus	19.2 (26)	1.8	0.6–5.5	0.29
Heatsynch	Proestrus	24.1 (29)	1.4	0.5–3.7	0.55
Ovsynch	Ovarian cyst	30.0 (20)	1.0	0.3–3.0	0.98
Heatsynch	Ovarian cyst	5.0 (20)	8.2	1.0–65.8	0.04

Table 4
Pregnancy rates on day 45, adjusted odds ratio (AOR), 95% confidence interval (CI) and levels of significance for the risk of non-pregnancy for cows in the Ovsynch and Heatsynch groups and at different stages of the estrous cycle, or presence of ovarian cysts

Variable		Pregnancy rate % (N)	AOR	95% CI	P value
Group	Ovarian classification				
Ovsynch	Diestrus	19.7 (81)	Referent	Referent	–
Heatsynch	Diestrus	16.7 (72)	1.5	0.6–3.5	0.36
Ovsynch	Metestrus	9.1 (22)	2.4	0.5–11.7	0.27
Heatsynch	Metestrus	44.4 (27)	0.3	0.1–0.8	0.01
Ovsynch	Proestrus	14.3 (35)	1.7	0.6–5.3	0.32
Heatsynch	Proestrus	15.8 (38)	1.5	0.5–4.6	0.42
Ovsynch	Ovarian cyst	26.1 (23)	0.9	0.3–2.7	0.86
Heatsynch	Ovarian cyst	8.0 (25)	4.2	0.8-20.1	0.07
Season					
August–September		19.9 (201)	0.5	0.2–1.2	0.12
October–December		16.8 (131)	Referent	Referent	–
Inseminator					
A		4.6 (22)	6.8	0.7–69.4	0.10
B		18.5 (108)	1.2	0.3–4.0	0.81
C		14.3 (28)	1.4	0.3–6.8	0.67
D		23.5 (68)	0.7	0.2–2.1	0.51
E		22.1 (68)	0.8	0.2–2.4	0.65
F		15.8 (38)	Referent	Referent	–

Administration of 1 mg of estradiol benzoate on day 13 after estrus synchronizes the ovarian follicular wave and subsequent estrus [32]; such a treatment in combination with a medroxyprogesterone acetate impregnated sponge increased the number of cows re-inseminated between days 18 and 25 after AI [33]. Insertion of the CIDR (progesterone

Table 5
Pregnancy rates on day 90, adjusted odds ratio (AOR), 95% confidence interval (CI) and levels of significance for the risk of non-pregnancy for cows in the Ovsynch and Heatsynch groups and at different stages of the estrous cycle, or presence of ovarian cysts

Variable		Pregnancy rate % (N)	AOR	95% CI	P value
Group	Ovarian classification				
Ovsynch	Diestrus	17.5 (80)	Referent	Referent	–
Heatsynch	Diestrus	15.3 (72)	1.1	0.5–2.7	0.79
Ovsynch	Metestrus	0.0 (22)	4.9	1.1–36.9	0.03
Heatsynch	Metestrus	32.0 (25)	0.4	0.1–1.1	0.08
Ovsynch	Proestrus	11.4 (35)	1.8	0.5–5.9	0.35
Heatsynch	Proestrus	15.8 (38)	1.2	0.4–3.6	0.71
Ovsynch	Ovarian cyst	21.7 (23)	0.7	0.2–2.4	0.62
Heatsynch	Ovarian cyst	4.2 (24)	6.0	0.7–44.7	0.09
Days in milk					
72–160		10.4 (77)	1.5	0.6–4.1	0.38
161–225		13.1 (84)	1.3	0.5–3.1	0.60
226–315		22.2 (81)	0.5	0.2–1.1	0.10
316–724		14.1 (85)	Referent	Referent	–

intravaginal insert) alone [34] or in combination with an estrogen injection either at CIDR insertion, removal, or 24 h after removal, can synchronize the return to estrus following a prior AI [31,35,36]. These programs require detection of estrus, and fertility has been reported to be normal [34,36], or decreased [33,35] when compared to cows re-inseminated at a spontaneous return to estrus.

Because estrus expression and detection are reduced in high producing lactating dairy cows [37], resynchronization of estrus needs to be combined with resychronization of ovulation and TAI. Considering that the stage of the estrous cycle affects the efficacy of TAI protocols [7,8], different strategies for resynchronization and TAI have been considered. Pregnancy rates were similar in cows with or without a CL at per rectum examination of the genital tract and then assigned to different protocols that combined estrus detection and TAI [10]. The use of ultrasonography permitted the initiation of GnRH for resynchronization at 7 days prior to a non-pregnancy diagnosis at 26–28 days after the previous AI [11–13] or to apply shortened protocols utilizing $PGF_{2\alpha}$ followed either by GnRH [14] or ECP [15,16] to induce ovulation for TAI.

In the present study, since there were a small number of cows in anestrus, only stages of the estrous cycle and presence of ovarian cysts were evaluated when the resynchronization protocol was initiated. There was a significant interaction between the Ovsynch and Heatsynch protocols and stages of the estrous cycle or presence of ovarian cysts. Cows in metestrus subjected to the Heatsynch protocol had an increased pregnancy rate. It was reported previously that metestrus stage is not ideal to initiate the Ovsynch protocol [7,8]. At the metestrus stage, a follicular wave has already been recruited and GnRH will fail to induce follicular turnover. Therefore, at the time of induced ovulation 48 h after $PGF_{2\alpha}$, the follicle may be aged and fertility reduced due to an extended period of dominance [38–40] as well as increased embryonic losses [41]. In the present study, a high proportion of cows in the metestrus stage assigned to the Heatsynch protocol were inseminated earlier at estrus on days 8 and 9, which may attenuate this adverse effect and increase fertility.

In contrast, cows with ovarian cysts had a higher pregnancy rate to the Ovsynch protocol. The positive feedback of estradiol on inducing the LH surge is compromised in cows with ovarian cysts [42–45]. Abnormal release of the GnRH surge from the hypothalamus is believed to be the cause of ovarian cysts in lactating dairy cows [46] and administration of GnRH induced an LH surge in cows with ovarian cysts [47]. Therefore, the Ovsynch protocol that includes injection of GnRH to induce ovulation may be more effective than the Heatsynch protocol, which attempts to induce hypothalamic release of GnRH via injection of ECP in cows with an impaired positive feedback to estradiol. In a previous study, Ovsynch generated an acceptable pregnancy rate in lactating dairy cows with ovarian cysts compared to cows with ovarian cysts inseminated at detected estrus or cows without ovarian cysts subjected to the Ovsynch protocol [26].

In conclusion, when cows were diagnosed non-pregnant by ultrasonography at 27 days post-AI to a previous service, resynchronization of ovulation and TAI with Ovsynch and Heatsynch protocols resulted in similar pregnancy rates. However, the Heatsynch protocol increased pregnancy rate for cows in metestrus at the time of initiation of the treatment regime, and the Ovsynch protocol was more effective for cows with ovarian cysts. The use of per rectum examination in combination with ultrasonography of the genital tract to determine stages of the estrous cycle, presence of ovarian cysts or anestrus may be valuable tools for selective resynchronization of non-pregnant cows at the time of pregnancy diagnosis.

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