ORIGINAL ARTICLE

Development of the Timed Re-Insemination (TRI-synch) program re-inseminating 24 days after the initial service in dairy cows

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Abstract

For the timed re-insemination at the minimal interbreeding interval, cows were treated with a progesterone (P_4)-releasing intravaginal device from Days 13–15 to 21 post-insemination (Day 0 = estrus), followed by plasma P_4 assay on Day 23 and then subjected to the Experiments 1 and 2. In Experiment 1, of 18 cows, 6 cows were determined as luteolysis with low (<1 ng/ml) plasma P_4 concentrations on Day 23 and ovulated on Days 24 (3 cows), 25 (1 cow), and 26 (1 cow) except a cow affected by ovarian quiescence. In Experiment 2, all cows were treated with GnRH on Day 23. Cows with low (<1 ng/ml) plasma P_4 concentrations on Day 23 were diagnosed as non-pregnant and subjected to the re-insemination in the morning of Day 24 irrespective of estrous signs. Of 36 cows, 15 cows were diagnosed as being non-pregnant on Day 23. Fourteen cows of the non-pregnant animals were re-inseminated in the morning of Day 24 irrespective of estrous signs and the pregnancy rate of re-insemination was 36%. The conception rates of initial and re-inseminations were 50% (18/36) and 36% (5/14), respectively. The overall pregnancy rate by adding the rates of initial and re-inseminations was 64% (23/36).

KEYWORDS
CIDR, cow, progesterone, synchronization, Timed-AI

1 | INTRODUCTION

The reproductive performance of dairy cows has been decreasing steadily over the past 3 decades. Current statistics show that the pregnancy rate per insemination, calving interval, and number of inseminations required for establishing pregnancy were 44%, 433 days, and 2.3 times, respectively (Ahmed et al., 2016). Currently, more than half of the inseminations resulted in failure. Since poor reproductive performance directly threatens the profitability of

dairy farms, several breeding programs for the improvement of reproductive efficiency have been introduced to the herd (Bisinotto & Santos, 2011; Mendonça et al., 2012; Pereira et al., 2013). These breeding programs aim to minimize the calving interval, which is accomplished by either improvement of the rate of pregnancy per insemination or shortening of the interbreeding interval when previous insemination has failed.

To minimize the interbreeding interval in cows in which previous insemination failed, several resynchronizing protocols have

been proposed (Bartolome et al., 2005; Pursley et al., 1995; Wijma et al., 2017, 2018). The principle of these programs is a combination of early pregnancy diagnosis around Days 25–30 post insemination (Day 0 = estrus) by ultrasonography and subsequent estrus synchronization by using a progesterone (P_4)-releasing intravaginal device (alias Controlled Internal Drug Release, CIDR) and/or prostaglandin $F_{2\alpha}$. However, since these programs might overlook the returning estrus around Day 21 post insemination, intensive studies are now ongoing to detect the pregnancy or non-pregnancy prior to the returning estrus (Gifford et al., 2007; Kizaki et al., 2013; Yoshino et al., 2018). Detecting the non-pregnancy without delaying returning estrus which is expected around Day 21 post insemination is regarded as a milestone. However, such a program for re-insemination after being diagnosed as non-pregnant is yet to be developed.

Kelley et al. (2016) reported a new protocol to resynchronize non-pregnant cows and heifers 21 days after the last insemination. In that study, inseminated cows were treated with CIDR on Days 13–20, and then subjected to pregnancy diagnosis by luteal ultrasonography on Day 20, and GnRH injection and re-insemination were carried out simultaneously on Day 21 when diagnosed as being non-pregnant. This study is groundbreaking since this procedure consists of both pregnancy diagnosis on Day 20 and Co-Synch on Day 21, and enables timed re-insemination at ordinary estrous intervals. As a result, the sensitivity, specificity, positive predictive value, and negative predictive value of the pregnancy diagnosis in cows on Day 20 were 100%, 43%, 43%, and 100%, respectively. The low positive predictive value (43%) means that more than half of the cows diagnosed as pregnant are non-pregnant resulting in the absence of re-insemination. Since structural luteal regression follows the decrease in P_4 production (Skarzynski et al., 2013), the results of an ultrasound examination on Day 20 might contain certain ambiguity for determining luteolysis. To improve the accuracy of luteal examination, functional analysis such as plasma P_4 measurement might be suitable. Therefore, in the present study, we aimed to perform accurate diagnosis of the expected pregnancy or non-pregnancy on Day 23 by assaying plasma P_4 concentrations in CIDR-treated cows.

This study aimed to establish a reliable program for the resynchronization of cows in which previous insemination had failed. We planned to re-inseminate cows within the regular estrous interval (up to 24 days) with a timed insemination procedure irrespective of estrous signs.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

A total of 87 lactating Holstein Friesian cows were used. The experiments were conducted from October 2015 to April 2017 on three commercial dairy farms in Takizawa City, Iwate Prefecture, Japan. The age, parity, daily milk yield, and days postpartum were 4.3 ± 2.1 years (ranging from 2 to 9), 2.6 ± 1.8 (ranging from 1 to 7), 31.0 ± 8.9 kg (ranging from 10 to 44.5), and 145.7 ± 78.5 days

(ranging from 68 to 434), respectively. Cows were maintained in tie-stall pens, milked twice daily, fed a mixture of forage and crops depending on their milk yield, and allowed permanent access to fresh water and mineralized salt. The experimental design of the present study was approved by the ethical review board of Iwate University (A201315) and informed consent was obtained from each farm. The day of estrus was defined as Day 0. Estrus was detected by the farm staff without a synchronization program. Estrus detection was carried by the observation of restlessness, bawling and discharge of clear mucus from the vagina. The timing of insemination was decided by the farm staff's judgment.

All cows were inseminated by a veterinarian on Day 0 or 1 with frozen semen collected from proven sires. On Days 13–15, CIDR (containing 1.9g of progesterone; Zoetis Japan, Tokyo, Japan) was inserted into all animals and withdrawn on Day 21.

2.2 | Ultrasonography

Transrectal B-mode ultrasonography (Tringa V linear with a 7.5 MHz ultrasound probe, Esaote Europe, Maastricht, Netherlands) was carried out on the day of CIDR insertion and Days 21–25. Ultrasonography examined the maximal cross-area of the corpus luteum by measuring major and minor axes of the images using the caliper function of the ultrasonography scanner. The luteal cross-area was defined as previously reported (Kot & Ginther, 1999) and calculated as follows: cross-area (cm^2) = $\pi\{(major\ axis\ (cm) \times minor\ axis\ (cm))/4\}$. When the luteal cross-area contained vacuoles, the net cross-area was calculated by subtracting the vacuolated area from the total. Pregnancy diagnosis was carried out on Days 28–30 by ultrasonography.

2.3 | Blood collection and plasma P_4 assay

Peripheral blood was collected from the coccygeal vein by using a heparinized vacuum collection tube (TERUMO, Tokyo, Japan) on the day of ultrasonography. Collected blood was placed on ice immediately after the collection and centrifuged at $1,600g$ for 15 min under refrigeration. Harvested plasma was stored at −30°C and used for the assay. Plasma P_4 assay was carried out by direct enzyme immunoassay according to a previous report (Kanazawa et al., 2016). Plasma samples or standards (25 μl, neat) were dispensed to a 96-well immunoplate (MaxiSorp; Thermo Fischer Scientific, Roskilde, Denmark) previously immobilized with anti-rabbit IgG antibody (Cappel, Solon, OH, USA). Anti-P_4 antiserum (Protein Purification Inc., Gunma, Japan) and P_4-carboxymethyloxime-horseradish peroxidase (HRP) conjugate (Cosmo Bio, Tokyo, Japan) were added to the well, incubated at room temperature for 3 hr, and then washed. Orthophenylenediamine as a substrate for HRP was added to the well and the plate was further incubated at room temperature for 30 min in the dark. Color reaction was terminated by sulfuric

acid and the absorbance (A_{490}) was measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The minimum detectable level, intra- and inter-assay coefficients of variation in P_4 assay were 0.037 ng/ml, 3.86% and 9.69%, respectively.

2.4 | Experimental design

2.4.1 | Experiment 1

A total of 18 cows were used in Experiment 1. All cows were inseminated on Day 0 or 1. In the morning of Day 13–15, CIDR was inserted into all animals and withdrawn in the morning of Day 21. Blood collection was carried out on the day of CIDR insertion and Days 21–25. Transrectal ultrasonography was carried out on the day of CIDR insertion and Days 21–26. During the experiment, blood collection and ultrasonography were carried out once a day in the morning (Figure 1). Cows were classified into two groups based on their plasma P_4 concentrations on Day 23. Cows with higher P_4 concentrations (≥ 1 ng/ml) and others (< 1 ng/ml) were designated as the corpus luteum-maintained (CL-M) and corpus luteum-regressed (CL-R) groups, respectively. The detection of returning estrus in the experimental animal was carried out by the daily observation of restlessness, bawling, discharge of clear mucus from the vagina, and vaginoscopy with the speculum. Ovulation following returning estrus was confirmed by daily ultrasonography.

2.4.2 | Experiment 2

A total of 69 cows were used in Experiment 2. All cows were inseminated on Day 0 or 1. In the morning of Day 13–15, CIDR was inserted into all animals and withdrawn in the evening of Day 21. Blood collection and transrectal ultrasonography were carried out on the day of CIDR insertion and Day 23.

On Day 21, cows were randomly assigned into two groups. Thirty-three cows, assigned to the estrus detection (ED) group, were monitored for signs of returning estrus. Estrus monitoring was carried out by the farm staff with twice-daily observation of restlessness, bawling, and discharge of clear mucus from the vagina. Cows exhibiting returning estrus were re-inseminated at the appropriate time. During the experiment, the results of plasma P_4 assay and ovarian ultrasonography in the ED group were blinded to the farm staff (Figure 2).
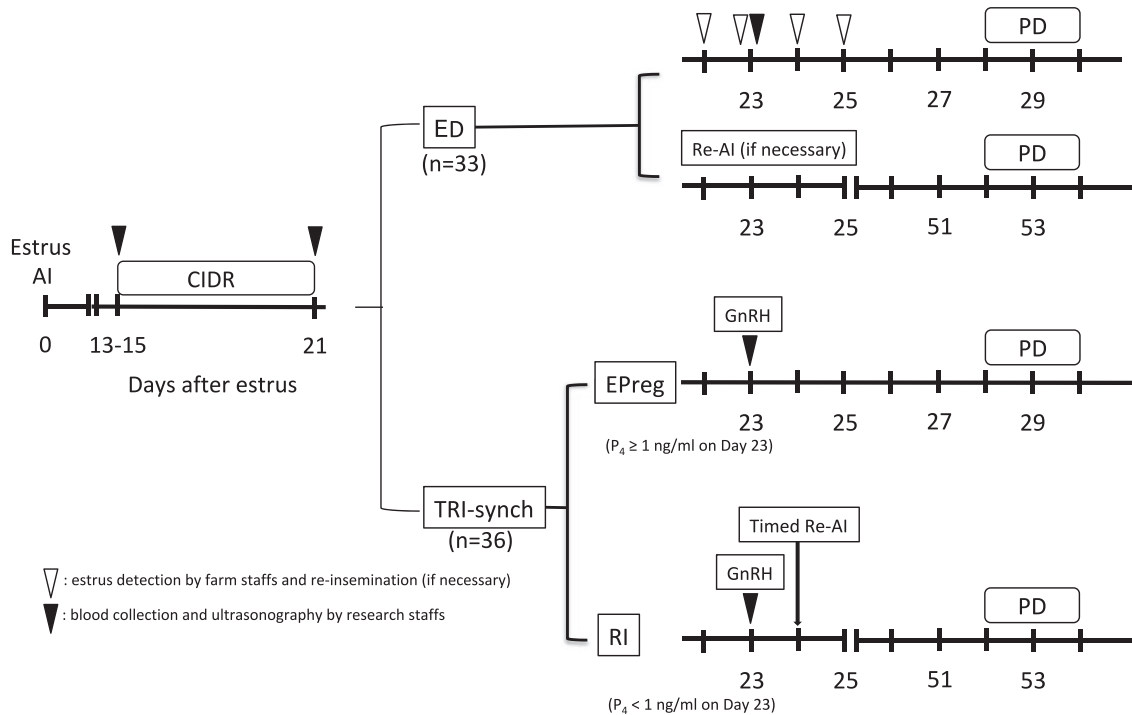
The remaining 36 cows, assigned to the TRI-synch group, were given GnRH agonist (100 µg fertirelin acetate; ASKA Pharmaceutical, Tokyo, Japan) in the morning of Day 23. On Day 23, blood samples were immediately brought to the laboratory, subjected to P_4 assay, and the results were obtained within several hours of the collection. In the afternoon of Day 23, cows in the TRI-synch group were classified into two subgroups based on their plasma P_4 concentrations on Day 23. Cows with higher P_4 concentrations (≥ 1 ng/ml) and others (< 1 ng/ml) were designated as the expected pregnancy (EPreg) and timed re-insemination (RI) subgroups, respectively. Cows in the RI subgroup were re-inseminated in the morning of Day 24 irrespective of estrous signs, whereas cows in the EPreg subgroup were left untreated (Figure 2).

2.5 | Statistical analyses

All data regarding the plasma P_4 concentrations and luteal cross-area are presented as means $\pm$ *SD*. All statistical analyses were carried out by using R i386 3.1.1 for Windows (R Development Core Team, Vienna, Austria). In Experiment 1, the data regarding changes in the plasma P_4 concentrations and luteal cross-area during the experiment between the groups were analyzed by one-way ANOVA followed by Tukey-Kramer's multiple comparison tests. In Experiment 2, the data regarding differences in the plasma P_4 concentrations between the groups were analyzed by Student's *t*-test. For analyzing the statistical significance of conception rates between the initial and re-inseminations, we used logistic regression by the method of maximum likelihood model with the conception rates as objective variable and the use of TRI-synch as explanatory variable. A probability value of < 0.05 was considered to be statistically significant.
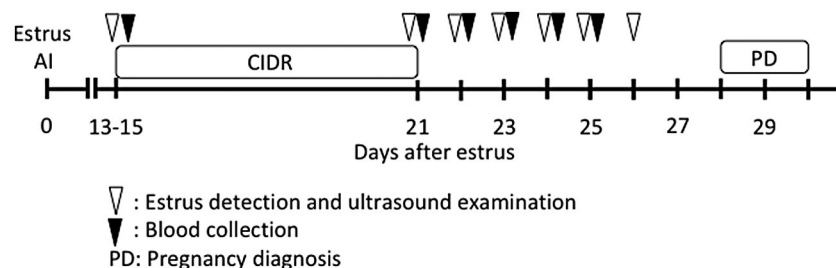
FIGURE 1 Schematic diagram of the Experiment 1. Cows were inseminated on Day 0 or 1 (estrus = Day 0). In the morning of Day 13–15, CIDR was inserted to all animals and withdrawn in the morning of Day 21. Blood collection was carried out on the day of CIDR insertion and Days 21–25. Transrectal ultrasonography was carried out on the day of CIDR insertion and Days 21–26. During the experiment, blood collection and ultrasonography were carried out once a day in the morning. Pregnancy diagnosis was carried out on Days 28–30 by ultrasonography

FIGURE 2 Schematic diagram of the Experiment 2. Cows were inseminated on Day 0 or 1. In the morning of Days 13–15, CIDR was inserted to all animals and withdrawn in the evening of Day 21. Blood collection and transrectal ultrasonography were carried out on the day of CIDR insertion and Day 23. On Day 21, cows were randomly assigned into two groups. Cows assigned to the estrous detection (ED) group were monitored for signs of returning estrus. Cows exhibited returning estrus were re-inseminated at the appropriate time. Cows, assigned to the TRI-synch group, were given GnRH agonist (100 μg fertilerin acetate, Asuka Pharmaceutical Co. Ltd., Tokyo, Japan) in the morning of Day 23. On Day 23, plasma P_4 concentrations were analyzed. In the afternoon of Day 23, cows in TRI-synch group were classified into two subgroups based on their plasma P_4 concentrations on Day 23. Cows with higher P_4 concentrations (>= 1ng/ml) and others (<1 ng/ml) were designated as the expecting pregnancy (EPreg) and timed re-insemination (RI) subgroups, respectively. Cows in the RI subgroup were re-inseminated in the morning of Day 24 irrespective of estrous signs, whereas cows in the EPreg subgroup were left untreated

3 | RESULTS

3.1 | Experiment 1

According to the plasma P_4 concentrations on Day 23, 12 and 6 cows were assigned to the CL-*M* ($P_4 \geq 1$ ng/ml) and CL-R ($P_4 < 1$ ng/ml) groups, respectively. In the CL-M group, 9 cows were diagnosed as being pregnant on Days 28–30, whereas 3 cows were not. Of 6 cows classified into the CL-R group, 4 and 1 cows exhibited returning estrus on Days 23 and 24, respectively. These cows ovulated on Days 24 (3 cows), 25 (1 cow), and 26 (1 cow) (Figure 3). The remaining 1 cow turned into the ovarian quiescence without exhibiting estrus and ovulation by Day 26.

On the day of CIDR insertion, there was no significant difference in the mean plasma P_4 concentrations between the CL-M and CL-R groups (3.21 ± 1.83 ng/ml and 2.34 ± 0.86 ng/ml, respectively). On Day 21, the mean plasma P_4 concentration in the CL-R group was decreased to 1.17 ± 1.18 ng/ml, but the difference between the groups was still not significant. After Day 22, the mean plasma P_4 concentration in the CL-R group was further decreased to 0.36 ± 0.63 ng/ml or less. The differences in the plasma P_4 concentrations between the groups were statistically significant after Day 22. By contrast, the mean plasma P_4 concentration in the CL-M group was maintained constantly at more than 3 ng/ml throughout the experiment (Figure 4A).

Similarly, there was no significant difference in luteal cross-areas between the CL-M and CL-R groups on the day of CIDR insertion (4.73 ± 1.96 cm^2 and 4.73 ± 1.15 cm^2, respectively). On Day 21, the mean luteal cross-area in the CL-R group was decreased to 3.21 ± 1.88 cm^2, but the difference between the groups was still not significant. On Day 22, the mean luteal cross-area in the CL-R group was further decreased to 2.35 ± 1.20 cm^2. The differences in the luteal cross-areas between the groups were statistically significant on Day 22. However, after Day 23, since the luteal cross-areas in four cows were hardly recognized in B-mode images, cross-area calculation was carried out with the remaining two cows. Therefore, statistical analyses beyond Day 22 were not performed, although the differences were apparent. By contrast, the mean luteal cross-areas in the CL-M group were maintained constantly at not less than 4.00 cm^2 throughout the experiment (Figure 4B).

3.2 | Experiment 2

In the ED group, according to the plasma P_4 concentrations on Day 23, 19 and 14 cows were assigned to the CL-*M* ($P_4 \geq 1$ ng/ml) and CL-R ($P_4 < 1$ ng/ml) subgroups, respectively. On Day 23, plasma
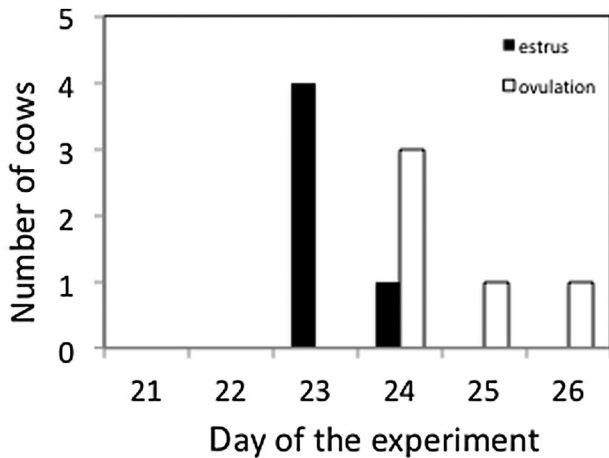
FIGURE 3 Distribution of the day of returning estrus (closed bar) and ovulation (open bar) in the Experiment 1. Cows were inseminated on Day 0 or 1 (estrus = Day 0). In the morning of Day 13–15, CIDR was inserted to all animals and withdrawn in the morning of Day 21. According to the plasma P_4 concentrations on Day 23, 6 cows were determined as luteolysis and assigned to CL-R group. Of 6 cows, 4 and 1 cows exhibited returning estrus on Days 23 and 24, respectively. These cows ovulated on Days 24 (3 cows), 25 (1 cow), and 26 (1 cow). The remaining 1 cow turned into the ovarian quiescence without exhibiting estrus and ovulation by Day 26

P_4 concentrations between the CL-M and CL-R subgroups were 3.55 ± 2.18 ng/ml and 0.13 ± 0.51 ng/ml, respectively. The differences in the plasma P_4 concentrations between the subgroups were statistically significant ($p < .01$). In the CL-M subgroup, 14 cows were diagnosed as being pregnant on Days 28–30, whereas 5 cows were not. No cows in the CL-R subgroups conceived by initial insemination.

Of the 14 cows classified into the CL-R subgroup, only 6 cows exhibiting returning estrus were detected and subjected to the re-insemination. The other 8 cows were overlooked for the returning estrus. The rate of re-insemination in the CL-R subgroup in ED group (re-inseminated/ returned new estrous cycle) was 43% (6/14). The conception rates of initial and re-inseminations were 42% (14/33) and 67% (4/6), respectively. The overall pregnancy rate in the ED group by adding the rates of initial and re-inseminations was 55% (18/33).

In the TRI-synch group, 21 cows were designated as the EPreg subgroup on Day 23 and 18 of them were diagnosed as being pregnant on Days 28–30, whereas 15 cows were designated as the RI subgroup. No cows in the RI subgroup conceived by the initial insemination. On Day 23, the mean plasma P_4 concentrations between the EPreg and RI subgroups were 3.38 ± 2.02 ng/ml and 0.05 ± 0.03 ng/ml, respectively. The differences in the plasma P_4 concentrations between the subgroups were statistically significant ($p < .01$). Fourteen cows in the RI subgroup were subjected to the re-insemination in the morning of Day 24; however, re-insemination was canceled in one cow due to it having ovulated on Day 24. The rate of re-insemination in the TRI-synch group (re-inseminated/ RI subgroup) was 93% (14/15). The conception rates of initial and re-inseminations were 50% (18/36) and 36% (5/14), respectively. The overall pregnancy rate in the TRI-synch group
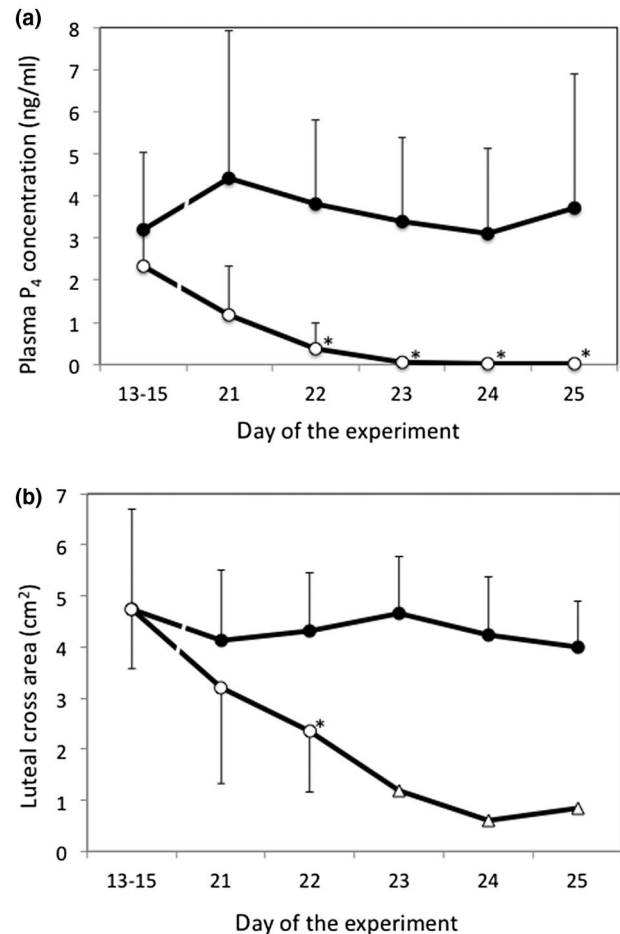
FIGURE 4 Temporal changes in the plasma P_4 concentrations (a) and luteal cross areas (b) in the Experiment 1. Results of CL-*M* (closed circle; $n = 12$) and CL-R (open circle; $n = 6$, and triangle; $n = 2$) groups are shown. Asterisks represent statistical significance ($p < .05$). In panel A, the *SD* values of Plasma P_4 concentrations beyond Day 22 in CL-R group were less than 0.03. Therefore, error bars are not seen in the figure. In panel B, since the luteal cross-areas in 4 cows were hardly recognized in B-mode images after Day 23, cross-area calculation was carried out with the remaining 2 cows (triangle). Therefore, statistical analyses beyond Day 22 were not performed

by adding the rates of initial and re-inseminations was 64% (23/36). The differences in the conception rates of initial insemination, re-insemination, and the combined overall were not significant between the groups. However, the rate of re-insemination was significantly greater ($p < .01$) in TRI-synch group than that in ED group (14/15 versus 6/14).

As the total results combining the ED and TRI-synch groups, the sensitivity, specificity, positive predictive value, and negative predictive value of the pregnancy diagnosis by plasma P_4 assay on Day 23 were 100%, 78%, 80%, and 100%, respectively (Table 1).

4 | DISCUSSION

The results of Experiment 1 showed that the administration of CIDR from Days 13–15 to 21 delayed the returning estrus in cows in which

	Pregnant (US, Day 28–30)	Non-pregnant (US, Day 28–30)	Total	
CL-M (Day 23) 1 ng/ml ≤ P4	32	8	40	PPV: 80%
CL-R (Day 23) P4 < 1 ng/ml	0	29	29	NPV: 100%
Total	32	37	69	
	Sens.: 100%	Spec.: 78%		

TABLE 1 Diagnostic accuracy of plasma P_4 assay on Day 23 for pregnancy outcome in the Experiment 2

Note: Cows with higher P_4 concentrations (≥1 ng/ml) and others (<1 ng/ml) were designated as the corpus luteum-maintained (CL-M) and corpus luteum-regressed (CL-R) groups, respectively. As the total results combining the ED and TRI-synch groups, the sensitivity (Sens.), specificity (Spec.), positive predictive value (PPV), and negative predictive value (NPV) of the pregnancy diagnosis by plasma P_4 assay on Day 23 are shown.

previous insemination had failed. Returning estrus in CL-R cows was concentrated on Days 23 and 24, as 2 or 3 days after CIDR removal. Ovulation occurred 1 or 2 days after the estrus. The day of estrus and ovulation partly deviated due to once-a-day clinical examination in this study. The consequence of returning estrus and ovulation in the present study agreed with those of the previous report (Chenault et al., 2003). Although within the limited number of animals, CIDR treatment from Days 13–15 to 21 appeared to interrupt the physiological consequence during luteolysis in non-pregnant animals. In Experiment 1, the mean plasma P_4 concentration in the CL-R group was 1.17 ± 1.18 ng/ml on Day 21. Our results suggest that the low but significant plasma P_4 concentration was still provided by CIDR during and after luteolysis. Plasma P_4 concentration around 1 ng/ml is sufficiently low allowing follicular turnover of the dominant follicles (Savio et al., 1993), but still too high for the induction of LH surge (Ginther et al., 2013). On Days 22 and 23, the plasma P_4 concentration in the CL-R group further decreased to 0.36 ± 0.63 ng/ml and 0.05 ± 0.03 ng/ml, respectively. Since the plasma P_4 concentration declines rapidly within several hours of CIDR removal (Cerri et al., 2009), the values on Days 22 and 23 appeared to be an endogenous secretion from the regressed CL, which allows pre-ovulatory LH surge.

Changes in the luteal cross-area in the CL-M and CL-R groups were comparable with those of plasma P_4 concentrations, respectively. The time difference between the functional and structural luteolyses indicated as the respective plasma P_4 concentrations and luteal cross-area (Sugino & Okuda, 2007) was not obvious in the present study probably due to once-a-day sampling. The results in Experiment 1 showed that more than half of the animals in the CL-R group returned to estrus on Day 23. In these cases, the induction of ovulation for timed re-insemination might be optimal in the evening of Day 22. Therefore, in Experiment 2, in order to achieve both complete luteolysis and uniformed timing of GnRH-induced ovulation, CIDR was removed in the evening of Day 21 and GnRH was injected in the morning of Day 23 in the TRI-synch group.

In the RI subgroup of the TRI-synch group in the Experiment 2, timed re-insemination in 1 of the 15 cows was canceled due to it having ovulated on Day 24, whereas 14 cows were re-inseminated as originally planned. Since the time interval between the GnRH administration and ovulation is 24–32 hr (Pursley et al., 1995), the induction of ovulation and timed re-insemination seemed to be well programmed in the present study.

In the TRI-synch group, although it was inherently unnecessary in the EPreg subgroups, all cows were given GnRH in the morning of Day 23. In the present study, the distribution of cows to the EPreg and RI subgroups depended on the results of plasma P_4 assay on Day 23. Since the plasma P_4 assay required several hours after blood collection, GnRH treatment had to be conducted in advance. One idea is that blood collection, plasma P_4 assay, and assignment to the subgroups are carried out on Day 22, and GnRH is injected only in the RI subgroup on Day 23. However, since plasma P_4 concentrations have not yet decreased to nadir in the RI subgroup, there is concern that plasma P_4 assay on Day 22 leads to a decrease in diagnostic accuracy. To omit GnRH treatment in the EPreg subgroup, it is desired to establish a real-time or cow-side pregnancy diagnosis method on Day 23. Among the many procedures that have been proposed for early pregnancy diagnosis in the cow, a milk P_4 qualitative stick (Ingenhoff et al., 2016) and luteal vasculature imaging with Doppler ultrasonography (Herzog et al., 2010; Kanazawa et al., 2016) might be promising.

In the present study, GnRH treatment in all cows in the TRI-synch group produced an unexpected index for pregnancy diagnosis on Days 28–30. Most of the pregnant cows in the TRI-synch group had twin corpora lutea both initially developed and GnRH induced; therefore, examination of the twin corpora lutea was practically helpful for pregnancy diagnosis.

The positive predictive value of plasma P_4 assay on Day 23 for pregnancy diagnosis was 80%, although the negative predictive value was 100%. Since the negative predictive value of plasma P_4 assay was 100%, plasma P_4 assay on Day 23 may have qualities to diagnose non-pregnancy in the TRI-synch program.

The estrous sign was detected in only 43% (6/14) of cows in the CL-R subgroup of the ED group and these were subjected to the re-insemination, although returning estrus was detected in 83% (5/6) of cows in the CL-R group of Experiment 1. In Experiment 1, estrus detection was the authors' mission in the present study, whereas in Experiment 2, estrus detection of the

ED group was carried out by the farm staff. In Experiment 1, we detected returning estrus by using rectal palpation, ultrasonography, and vaginoscopy in addition to general inspection of the cow. However, in Experiment 2, the farm staff tried to detect returning estrus by general inspection without rectal palpation, ultrasonography, and vaginoscopy. It has been reported that the average rate of estrus detection in US dairy herds was usually less than 50% and low detection rate was a major affecting reproductive performance (Lopez et al., 2004). In tie-stall pens, estrus detection of dairy cows was reported as being further difficult (Sakaguchi et al., 2007; Sumiyoshi et al., 2014). Therefore, timed re-insemination seems to have tremendous advantages in terms of efficient recruitment for re-insemination in cows in which previous insemination had failed.

In CL-M cows of the ED group and EPreg cows of the TRI-synch group, 8 cows each were diagnosed as being non-pregnant on Days 28–30 suggesting the incidence of pregnancy loss. Results in the present study suggest that the evaluation of luteal function by plasma P_4 assay on Day 23 is not able to forecast the occurrence of pregnancy loss in advance. Several procedures for extra-early pregnancy diagnosis in the cow have been proposed (Gifford et al., 2007; Pohler et al., 2017; Schanzenbach et al., 2017; Yoshino et al., 2018); however, *pre-hoc* diagnosis of pregnancy loss has not been established yet. Therefore, the positive predictive value never increases up to 100%.

The TRI-synch protocol, a timed re-insemination program for cows in which previous insemination failed, consists of CIDR withdrawal on Day 21, pregnancy diagnosis and GnRH treatment on Day 23, and timed re-insemination on Day 24. Several studies have been reported regarding the ovulation resynchronization program following non-pregnancy diagnosis in dairy cows (Sinedino et al., 2014; Spencer et al., 2018; Wijma et al., 2018). In these studies, non-pregnancy diagnoses have carried out on Day 28 (Sinedino et al., 2014), Day 32 (Wijma et al., 2018), and Day 37 (Spencer et al., 2018), and following re-inseminations were done further 3–8 days later. In the present study, TRI-synch program enabled both non-pregnancy diagnosis on Day 23 and timed re-insemination on Day 24 indicating the more time saving compared to the previous studies.

There are few studies on the resynchronization program prior to non-pregnancy diagnosis (Kelley et al., 2016). Kelley et al. (2016) have reported the timed re-insemination program for cows in which previous insemination failed with a combined use of CIDR, ultrasonography, and GnRH treatment. Cows were inseminated, administrated CIDR on Days 13–20, and underwent ovarian ultrasonography on Day 20, and GnRH treatment and re-insemination on Day 21 if luteolysis was confirmed by ultrasonography on Day 20. The conception rates (pregnancy per insemination) of initial and re-inseminations were reported as 30.3% and 24.2%, whereas in the present study, the pregnancy rates of initial and re-inseminations were 50% and 36%, respectively. Additionally, there is a difference in the positive predictive value for pregnancy between the previous report (Kelley et al., 2016) and the present study (43% versus 80%,

respectively), although the negative predictive values for pregnancy in both studies were 100%.

Pregnancy diagnosis was achieved on Day 20 based on the luteal measurement by Kelley et al. (2016) and it is suggested that since structural luteolysis in non-pregnant animals is delayed compared with the decrement of plasma P_4 concentration (Skarzynski et al., 2013), luteal measurement on Day 20 tends to overlook the non-pregnant animals. In the present study, pregnancy diagnosis by plasma P_4 assay on Day 23 might contribute to a relatively higher positive predictive value for pregnancy. However, it has also been reported that the longer CIDR treatment extends the follicular wave and has adverse effects on the pregnancy rate in synchronized cycles (Odde, 1990). In Experiment 2, although there was no significant difference in the conception rates between the initial and re-inseminations, the conception rate of re-insemination was inferior to that of the initial. In Experiment 1, CIDR was removed on Day 21 and subsequent ovulations in non-pregnant animals were observed on Days 24 to 26. The ovulated follicles were the same dominant follicles observed on Day 21, suggesting the extended age of oocytes in the dominant follicles. It has been reported that the pre-ovulatory oocytes exposed to a longer period of follicular phase could lead to the reduced fertility (Wiltbank et al., 2006). Results in the present study imply the improved conception rate of re-insemination after shorter period of CIDR treatment. The most optimal timing of CIDR removal and pregnancy diagnosis should be further elucidated.

5 | CONCLUSIONS

This study established a reliable resynchronization program for dairy cows in which previous insemination had failed without any detrimental effect on the result of the initial insemination. In the present study, pregnancy diagnosis was carried out on Day 23, approximately 80% of non-pregnant animals were subjected to the re-insemination on Day 24 irrespective of estrous signs and the pregnancy rate of re-insemination was 36%. The overall pregnancy rate was increased from 50% for initial insemination alone to 64% for the combination of initial and re-inseminations. The TRI-synch program is expected to be a practical tool for the rapid increase in cumulative pregnancy rates and the reduction in the interbreeding interval in the herd.

CONFLICT OF INTEREST

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the study.

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