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—Original Article—

Effects of human chorionic gonadotropin and intravaginal progesterone device treatment after artificial inseminations on the reproductive performance of normal and repeat breeder lactating dairy cows

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Abstract. We examined the effects of human chorionic gonadotropin (hCG) treatment on Day 5 (Day 0 = day of artificial insemination: AI) and intravaginal progesterone device (IVPD) treatment from Day 5 to 19 on the conception and detection rates of return to estrus (re-estrus) in lactating dairy cows. A total of 306 cows from a commercial dairy farm were divided into the following three groups on Day 5: non-treatment group (n = 128), untreated; hCG group (n = 71), 3,000 IU hCG was administered (intramuscularly); IVPD group (n = 107), IVPD was inserted into the vagina from Day 5 to 19. Re-estrus detection was performed up to Day 25. Pregnancy was diagnosed by rectal palpation between Day 50 and 60. There was an interaction between treatment and AI number ($P < 0.01$) on the conception rate of first-AI. For cows with more than three AIs, the IVPD treatment (66.7%) was more effective than the non-treatment (23.1%) ($P < 0.05$). The re-estrus detection rate was significantly ($P < 0.05$) higher in the IVPD group (60.7%) than that in the non-treatment group (41.4%) and tended ($P < 0.1$) to be higher than that in the hCG group (37.8%). Our results suggested that the conception rate can be improved by IVPD treatment, especially in cows with more than three AIs. In addition, IVPD treatment can induce higher estrus expression up to 25 days after AI in non-pregnant cows.

Key words: Conception rate, Cow, Estrus detection rate, hCG, Intravaginal progesterone device

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The improvement and maintenance of high reproductive performance in lactating dairy cows are important for the dairy industry [1]. However, one of the factors that can decrease reproductive performance is the existence of repeat breeder cows, which are defined as cows that do not get pregnant after three artificial inseminations (AIs) without any detectable abnormalities in their genital tracts and with apparently normal estrous cycles [2]. Although the causes of repeat breeder cows are various, such as subdued luteinizing hormone [3], lower oocyte competence [4], and alteration of endometrial function [5], lower plasma progesterone (P_4) concentrations after AI is one of the causes of repeat breeder dairy cows [6]. To increase plasma P_4 concentrations for higher fertility after AI, human chorionic gonadotropin (hCG) [7–10] or intravaginal P_4 device (IVPD) [11–13] treatment during the early luteal phase (3–10 days after AI) have been used in many studies. hCG has a luteinizing hormone-like effect in cattle [14] and induces the ovulation of the dominant follicle (DF) and forms an accessory corpus luteum (CL), with a subsequent increase of plasma P_4 concentration in cattle [10]. Treatment of IVPD increases plasma P_4 concentration immediately after insertion into the vagina [15]. Although plasma P_4 level gradually decreases after some time, it maintains a certain level as the luteal phase beyond two weeks [16]. In addition, plasma P_4 concentration rapidly decreases to the basal level when the IVPD is removed [17]. Although the utility of hCG [18] and IVPD [19] treatments for repeat breeder cows have been investigated after the early luteal phase, it is also important to compare the effects of IVPD and hCG treatments on the conception rate of repeat breeder cows because clinicians need to know which treatment is more effective for repeat breeder cows in the clinical situation. Although previous study compared the effect of hCG and IVPD treatments on the conception rates in repeat breeder cows [20], it has not been well compared the effects of IVPD and hCG treatments on the conception rates in normal and repeat breeder cows.

The estrus detection rate is one of the most important factors to improve the reproductive performance of non-pregnant cows [21]. High-yield lactating dairy cows have decreased expression and duration of estrus [22], which can lead to a lower estrus detection rate. To improve the synchronization of the return to estrus (re-estrus) and increase the re-estrus detection rate in non-pregnant cows, IVPD was inserted from 14 to 21 days after AI [23, 24]. However, the conception

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rate of re-estrus was lower when IVPD was removed on 21 and 22 days compared to 19 and 20 days after AI [24]. From these results, the earlier removal of IVPD might achieve a sufficient conception rate for AI of re-estrus. On the other hand, hCG treatment 5 days after estrus have more three waves and the third wave DF ovulate in heifers [25] and cows with three waves have longer estrous cycles than those with two waves in lactating dairy cows [26]; therefore, the timing of re-estrus might be different between non-treatment and hCG treatment, which might affect the re-estrus detection rate. However, the re-estrus detection rate has not been thoroughly examined after AI with hCG treatment during the early luteal phase.

Therefore, the objective of the present study was to evaluate the effects of hCG treatment at 5 days and IVPD treatment from 5 to 19 days after AI (first-AI) on the conception rate, detection rate of re-estrus, conception rate of re-estrus (second-AI), and cumulative pregnancy rate (first + second-AI) in normal and repeat breeder lactating dairy cows. This research will introduce an effective treatment option for higher conception and re-estrus detection rates in repeat breeder lactating dairy cows.

Materials and Methods

Animals and management

Postpartum lactating Holstein dairy cows (n = 306) at a commercial dairy farm in Hokkaido, northeast Japan, were used for this study (Parity: 2.6 ± 1.5, Days in milk: 151.0 ± 81.8, Previous AI number: 2.3 ± 2.0; mean ± SD). The trial was conducted from May 2009 to February 2011. All cows underwent regular estrous cycles and were evaluated as clinically healthy during the breeding period. Cows did not get pregnant after three AIs without detectable abnormalities in their genital tracts were defined as "repeat breeder cows". The cows were kept in a free-stall barn and fed a total mixed rations diet consisting of corn silage, grass silage, soybean meal, corn grain, and concentrate, with free access to water. All cows were milked thrice daily. The mean annual milk production during the study period was 10,350 kg per cow. A voluntary waiting period was set at 40 days after parturition. The experimental procedures complied with the Guide for the Care and Use of Agricultural Animals of Obihiro University.

Experimental design

Cows were inseminated (first-AI) after spontaneous estrus which was detected by visual observations by farm staff including standing heat, mounting to other cows, sniffing the vagina of other cow, swelling or hyperemia of the vagina, and mucous from the vagina. In addition, estrus was evaluated by rectal palpation to confirm the preovulatory follicle and regressed CL by artificial inseminator or veterinarian. On Day 5 (day of first-AI = Day 0), the cows were divided into the following three groups: non-treatment group (n = 128), which received no treatment; hCG group (n = 71), 3,000 IU hCG (Gestron 1500; Kyoritsu Seiyaku, Tokyo, Japan) was administered intramuscularly; IVPD group (n = 107): controlled internal drug release (CIDR: CIDR 1900; Zoetis Japan, Tokyo, Japan) insertion from Day 5 to 19. To decrease bias, we divided the groups by parity (1, 2, and ≥ 3) and AI number (≤ 3, > 3) blocked by each month. Pregnancy was determined by rectal palpation between Day 50 and 60. If estrus was detected before the pregnancy diagnosis, it was concluded that the cow was not pregnant. The conception rate was calculated by dividing the number of pregnant cows by the total number of cows enrolled in the study. Re-estrus was detected by visual observation until the next estrus was observed in the non-pregnant cows from 14 days after the first-AI. When estrus was detected up to Day 25, AI (second-AI) was conducted, and pregnancy was determined by rectal palpation 50 to 60 days after the second-AI. Figure 1 shows a schematic diagram of the experimental model.

We collected blood samples from the tail vein with a heparinized tube (Venoject II, VP-H100K, Terumo, Tokyo, Japan) on Day 5 and 6 in the non-treatment (n = 8), hCG (n = 7), and IVPD (n = 6) groups for measuring plasma P_4 concentration. In addition, blood samples were collected from cows with more than three AIs, and we divided randomly to the non-treatment, hCG, and IVPD groups. Blood samples were collected immediately before treatment on Day 5 and were collected again 22–26 h after the first blood sample collection on Day 6. Blood was separated by centrifugation (2000 × *g* for 20 min at 4°C) and frozen at −30°C until analysis.

Plasma P_4 concentration assay

Plasma P_4 concentrations were determined in duplicate using a double-antibody enzyme immunoassay, according to previously established procedures [27]. Assays were performed after extraction with diethyl ether. The recovery rate of P_4 was 83%. The standard curve ranged from 0.05 to 50 ng/ml. The ED50 of the assay was 2.2 ng/ml. The intra- and inter-assay CVs were 6.8% and 11.4%, respectively.

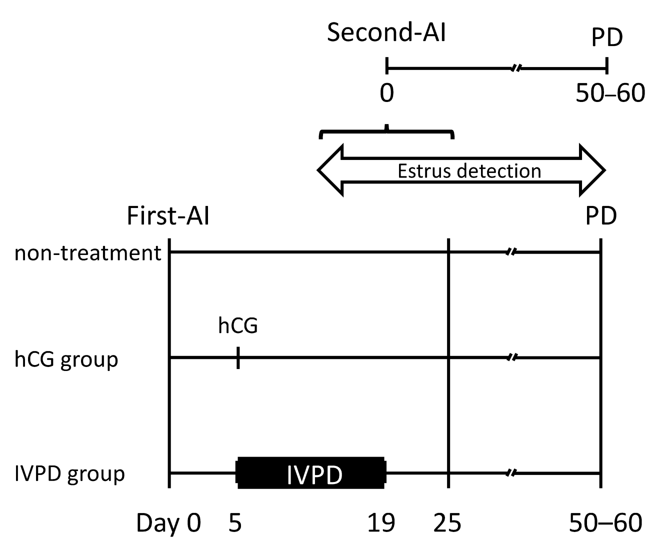

Fig. 1. Schematic diagram of the experimental model. The day of artificial insemination [first-artificial insemination (AI)] was defined as Day 0. Untreated was defined as "non-treatment". Human chorionic gonadotropin (hCG) (3,000 IU) was treated intramuscularly on Day 5 in "hCG group". Intravaginal progesterone device (IVPD) was inserted from Day 5 to 19 in "IVPD group". Estrus detection was performed from 14 days after first-AI. AI was performed when the estrus was detected up to Day 25 (second-AI). Pregnancy was determined by rectal palpation between 50 and 60 days after AI. PD, Pregnancy diagnosis.

Statistical analysis

All statistical analyses were performed using EZR (version 1.50, Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions that are frequently used in biostatistics [28].

Data of plasma P_4 concentration changed from Day 5 to 6 and days of re-estrus from first-AI were confirmed to follow a normal distribution by Shapiro-Wilk normality test and were analyzed by Bartlett's test to verify homogeneity of variances. From the results of Bartlett test's, variances were not equal among the three treatment groups in both plasma P_4 concentrations change and days of re-estrus from first-AI. Therefore, Kruskal-Wallis test was used to determine the main effect of treatment (non-treatment *vs*. hCG *vs*. IVPD). When a treatment effect was significant, Holm's test was used as a multiple comparison test to detect significant differences among treatment groups.

Binary outcomes (conception rate of first-AI, re-estrus detection rate, conception rate of second-AI, and cumulative pregnancy rate [proportion of pregnant cows combined of first- and second-AI]) were analyzed using multiple binary logistic regression. Pregnancy status (non-pregnant; 0, pregnant; 1) and re-estrus detection status (non-detected; 0, detected; 1) were analyzed as a dependent variable. To investigate the efficiency of re-estrus detection, re-estrus detected up to Day 25 was defined as "detected" and others were defined as "non-detected". The independent variables were treatment (non-treatment, hCG, and IVPD), parity (< 3, ≥ 3), days in milk at first-AI (< 150 days, ≥ 150 days), AI number (≤ 3, > 3) and the interactions between treatment and each independent variable were incorporated into the logistic regression model. We classified parity and days in milk into two groups with the mean. The final model for each outcome was selected by backward elimination of the independent variables with $P > 0.20$ and determination for the lowest value for the Akaike information criterion. Treatment was forced into all models. Final selected variables for the logistic regression model were treatment, AI number, and the interaction of treatment and AI number for conception rate of first-AI, and treatment, days in milk at first-AI, and AI number for re-estrus detection rate, and treatment and days in milk at first-AI for conception rate of second-AI, and treatment, AI number, and the interaction of treatment and AI number for cumulative pregnancy rate. Because of the interaction of treatment and AI number was significant, the conception rate of first-AI and the cumulative pregnancy rate among treatments by AI number groups were compared using chi-square test with Holm's adjustment for multiple comparison test as a post hoc test. In the model of the re-estrus detection rate, because the factor of treatment was significant, the re-estrus detection rate was assessed by chi-square test with Holm's adjustment for multiple comparison test as a post hoc test after multiple logistic regression analysis.

A P value of < 0.05 indicated a significant difference, and a P value of < 0.1 indicated a tendency. Data of plasma P_4 concentration change are presented as the mean ± SEM.

Results

Plasma P_4 concentration change from Day 5 to 6 among treatments

Analysis results the plasma P_4 concentration changes are shown in Fig. 2. The treatment effect was significant ($P < 0.001$). Plasma P_4 concentration change from Day 5 to 6 in the IVPD group was significantly higher than that in the non-treatment group ($P < 0.05$) and tended to be higher than that in the hCG group ($P < 0.1$). Plasma P_4 concentration change from Day 5 to 6 in the hCG group tended to be higher than that in the non-treatment group ($P < 0.1$).

Factors affecting the conception rate of first-AI

The logistic regression revealed that treatment ($P < 0.1$), AI number ($P < 0.05$), and their interaction ($P < 0.01$) were associated with the conception rate of first-AI. The conception rate of first-AI in the IVPD group with > 3 AIs was higher than that in the non-treatment with > 3 AIs ($P < 0.05$); however, there were no differences among the other subgroups (Table 1).

Days in milk at AI, parity, the interaction of treatment and days in milk at AI, and the interaction of treatment and parity were eliminated from the final model.

Days of re-estrus from first-AI in the non-treatent, hCG, and IVPD groups in non-pregnant cows

Distributions of the detection day of re-estrus are shown in Fig. 3. Analysis of the days of re-estrus from first-AI is shown in Table 2. The treatment effect was significant ($P < 0.001$). Days of re-estrus from first-AI in the IVPD group were significantly shorter than those in the non-treatment group ($P < 0.01$) and hCG groups ($P < 0.05$).

Factors affecting the re-estrus detection rate in non-pregnant cows

The logistic regression revealed that treatment ($P < 0.05$) was

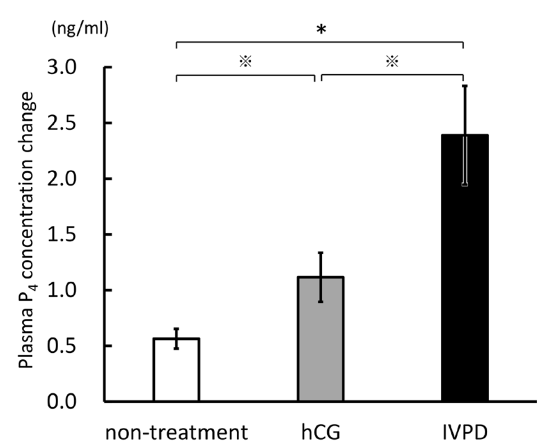

Fig. 2. Plasma progesterone (P_4) concentration change from Day 5 to 6 in the non-treatment (n = 8), human chorionic gonadotropin (hCG) (n = 7), and intravaginal progesterone device (IVPD) (n = 6). groups Different superscripts indicate difference, ※ $P < 0.1$, * $P < 0.05$. Data are shown as mean ± SEM.

Table 1. Effect of the interaction of artificial insemination (AI) number and treatment on the conception rate of first-AI and the cumulative pregnancy rate in the non-treatment, human chorionic gonadotropin (hCG), and intravaginal progesterone (P_4) device (IVPD) groups

Parameter	AI number	Group		
		Non-treatment [1]	hCG [2]	IVPD [3]
First-AI conception rate (%)	≤ 3	34.3 [ab] (35/102)	37.3 [ab] (22/59)	41.3 [ab] (33/80)
	> 3	23.1 [a] (6/26)	33.3 [ab] (4/12)	66.7 [b] (18/27)
Cumulative pregnancy rate (%)	≤ 3	43.1 [ab] (44/102)	42.4 [ab] (25/59)	56.3 [ab] (45/80)
	> 3	30.7 [a] (8/26)	75.0 [ab] (9/12)	66.7 [b] (18/27)

[1] Non-treatment: Untreated after first-AI. [2] hCG: 3,000 IU hCG was administered intramuscularly at 5 days after first-AI. [3] IVPD: IVPD was inserted from 5 to 19 days after first-AI. Different superscripts indicate difference within the same parameter. a, b: P < 0.05.

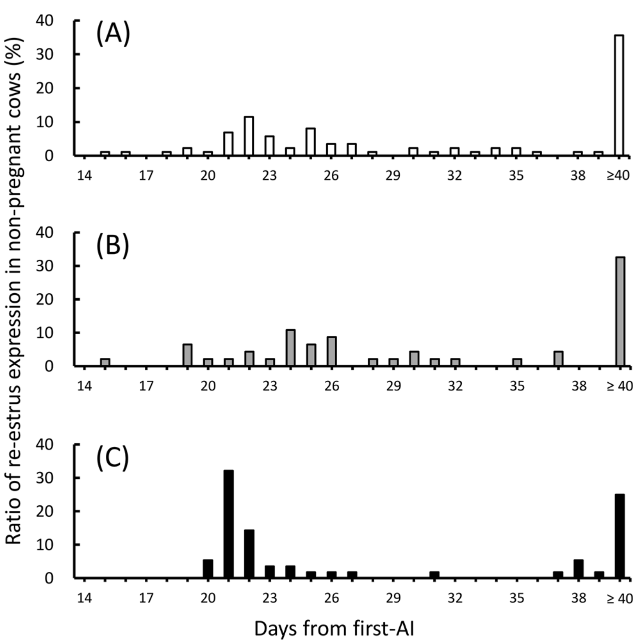

Fig. 3. Distribution of the timing of re-estrus expression in the non-treatment (A), human chorionic gonadotropin (hCG) (B), and intravaginal progesterone device (IVPD) (C) groups.

associated with the re-estrus detection rate. The re-estrus detection rate in the IVPD group was significantly higher than that in the non-treatment group (P < 0.05) and tended to be higher than that in the hCG group (P < 0.1) (Table 2).

Days in milk at first-AI and AI number were not associated with the re-estrus detection rate. Parity, the interaction of treatment and days in milk at AI, the interaction of treatment and parity, and the interaction of treatment and AI number were eliminated from the final model.

Factors affecting the conception rate of second-AI

The logistic regression revealed that treatment and days in milk at AI were not associated with the conception rate of second-AI. The conception rates of second-AI in the non-treatment, hCG, and IVPD groups are shown in Table 2. Parity, AI number, the interaction of treatment and days in milk at AI, the interaction of treatment and parity, and the interaction of treatment and AI number were eliminated from the final model.

Factors affecting the cumulative pregnancy rate

The logistic regression revealed that AI number (P < 0.05) and the interaction of treatment and AI number (P < 0.05) were associated with the cumulative pregnancy rate. The cumulative pregnancy rate in the IVPD group with > 3 AIs was higher than that in the non-treatment with > 3 AIs (P < 0.05); however, there were no difference among other sub groups (Table 1).

Treatment was not associated with the cumulative pregnancy rate. Days in milk at AI, parity, the interaction of treatment and days in milk at AI, and the interaction of treatment and parity were eliminated from the final model.

Discussion

The present study revealed that IVPD treatment from 5 to 19 days after AI increased the conception rate of first-AI, and the cumulative pregnancy rate in repeat breeder lactating dairy cows (AI number with more than three); on the other hand, the re-estrus detection rate was higher in IVPD treatment regardless of the AI number. However, hCG treatment did not affect the conception rate of first-AI, re-estrus detection rate, and cumulative pregnancy rate in the present study.

Early increase and higher level of plasma P_4 concentrations lead well development of the embryo and a higher secretion of interferon-tau [29]. Plasma P_4 concentration change from Day 5 to 6 in the IVPD group was significantly higher than that in the non-treatment group and tended to be higher than that in the hCG group in the present study. Plasma P_4 concentration increases by IVPD from 5

Table 2. Median days of re-estrus from first-artificial insemination (AI), re-estrus detection rate, and second-AI conception rate in the non-treatment, human chorionic gonadotropin (hCG), and intravaginal progesterone (P_4) device (IVPD) groups

Parameter	Non-treatment [1]	hCG [2]	IVPD [3]
Median days of re-estrus from first-AI (Lower quartile, Upper quartile)	30.0 [a] (22.5, 48.0)	29.0 [a] (24.0, 44.0)	22.0 [b] (21, 38.5)
Re-estrus detection rate (%)	41.4 [aA] (36/87)	37.8 [abA] (17/45)	60.7 [bB] (34/56)
Second-AI conception rate (%)	30.6 (11/36)	47.1 (8/17)	35.3 (12/34)

[1] Non-treatment: Untreated after first-AI. [2] hCG: 3,000 IU hCG was administered intramuscularly at 5 days after first-AI. [3] IVPD: IVPD was inserted from 5 to 19 days after first-AI. Different superscripts indicate difference within the same row. A, B: $P < 0.1$, a, b: $P < 0.05$.

days after estrus advances the endometrial gene expressions related triglyceride synthesis or glucose metabolism and uptake in cattle [30, 31]. Because of fast and higher increase of plasma P_4 concentration in the IVPD group, endometrium gene expression, such as the association of energy sources or contributors to histotroph, might enhance embryo development and interferon-tau secretion. Although hCG treatment could increase plasma P_4 concentration, a moderate increase during the early luteal phase after hCG treatment might not enhance embryo development compared to IVPD treatment. In the present study, IVPD treatment had a greater effect on increasing the conception rate in cows with more than three AIs. Although the causes of repeat breeder cows were multifactorial and various treatment options for repeat breeder cows were investigated, parthenogenetic embryo transfer following AI increases the conception rate in repeat breeder lactating dairy cows in a recent study [32]. Parthenogenetic embryos show higher interferon-tau mRNA expression and induce higher interferon-tau stimulated gene mRNA expression in the white blood cells of cows that was transferred parthenogenetic embryo in the uterus [32]. From these results, one of the causes of infertility in the repeat breeder cows might have been a shortage of interferon-tau secretion from the embryo. Therefore, it was speculated that IVPD treatment that induced greater embryo development with higher interferon-tau secretion might have contributed to an increase in the conception rate in repeat breeder cows in this study. The effects of hCG treatment 5 days after AI on fertility are not consistent between studies [9, 10]. From a recent study, hCG treatment 5 days after AI have a beneficial effect on fertility only when the first follicular wave DF (first-wave DF) developed ipsilaterally to the CL in the ovary in lactating dairy cows [33]. In this study, although we did not evaluate the locational relationship of the first-wave DF and CL, it is still necessary to confirm the location of the first-wave DF and CL for increasing the conception rate using hCG.

The estrus detection rate of non-pregnant cows was higher in the IVPD group, due to the tight synchronization during 1–3 days after removal of IVPD (Fig. 3); however, the conception rate of the second-AI was similar to that of the non-treatment and hCG groups. In a previous study, insertion of IVPD from 14 to 21 days after AI improves the synchrony of re-estrus during 1–3 days after IVPD removal with slightly reduced the AI conception rate of re-estrus [23]. Another study also reported that the estrus detection rate was higher after IVPD removal at 21 days compared to 19 days; however, removal of IVPD between 19 and 20 days after AI increases the conception rate of re-estrus compared to doing so between 21 to 22 days [24]. It is estimated that lower conception rate with removal between 21 to 22 was caused by the development of persistent follicles resulting of extended insertion of IVPD [34]. From these results, it can be concluded that to prevent lower conception rate, it is favorable to remove IVPD 19 days after first-AI. As a result, the conception rate of second-AI was similar to those of the non-treatment and hCG, groups and the re-estrus detection rate of the IVPD groups was nearly 1.5 times higher than those of the non-treatment and hCG groups. The higher estrus detection rate in the IVPD group might have been caused by tightly synchronized estrus; more than 50% of non-pregnant cows were in estrus during 3 days after removal. The cause of this tightly synchronized estrus was not clarified in the present study. A previous study showed that 19% of non-pregnant lactating dairy cows delay the onset of luteal regression, and these non-pregnant cows are diagnosed with the presence of an embryo by pregnancy-specific protein B at 24 days after AI [35]. In other words, there are a constant number of non-pregnant cows with delaying the onset of luteal regression because of early embryo loss in lactating dairy cows. From the results of the present study, it can be speculated that IVPD treatment from 5 days after AI might enhance embryo development, especially for lower quality embryos, and prevent embryo mortality. As a result, proportion of non-pregnant cows with early embryo loss might decrease and the proportion of non-pregnant cows with regular luteal regression timing might increase; therefore, proportion of re-estrus occurring around 21 days after AI might increase after IVPD treatment. Another possible cause of tightly synchronized estrus was that IVPD treatment suppressed the estrus expression of non-pregnant cows with regressed CL earlier than 19 days after AI. Because some of the non-pregnant cows in the non-treatment and hCG groups expressed estrus until 19 days after AI (Fig. 3), some of non-pregnant cows were already regressed CL and suppressed estrus expression until IVPD removal. Therefore, it was speculated that the non-pregnant cows with early luteolysis expressed estrus during 1–3 days after IVPD removal, and estrus expression might be tightly synchronized in the IVPD group. However, we need further research whether the cause of the tightly synchronized estrus expression was brought by lower embryo mortality after maternal recognition period in the IVPD group than that in the non-treatment or/and suppressed the estrus expression of non-pregnant cows with earlier luteolysis by IVPD.

The higher conception rate of first-AI and higher re-estrus detection rate led to a higher cumulative pregnancy rate in the IVPD group, especially in repeat breeder cows. Therefore, this treatment could be one of an effective strategy for the reproductive management of repeat breeder cows.

In conclusion, the present study demonstrated that due to higher conception and re-estrus detection rates, IVPD treatment from 5 to 19 days after AI had a beneficial effect on the reproductive management of lactating dairy cows. In addition, the effect of IVPD treatment on the conception rate was more effective in repeat breeder cows which were inseminated more than three times.

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