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Sustained high progesterone concentrations during estradiol-progesterone based estrus synchronization protocol in Japanese Black cows affects fertility by influencing preovulatory follicle size and its ovulation

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Abstract

Preovulatory follicle (POF) size during estrus synchronization has been reported as one of the factors affecting conception rate in cattle. In present study, to determine the effects of POF size on the fertility of Japanese Black cows, relationship between POF size and conception rates and the effects of progesterone (P_4) concentration on POF size were examined. An intravaginal progesterone-releasing device (CIDR) insertion and estradiol benzoate (EB) injection were applied to cows (day 0). At CIDR removal (day 8), the cows were received prostaglandin $F_{2\alpha}$ and subsequently artificially inseminated between days 10 and 11, after EB administration (day 9). The cow that did not ovulate within 3 days after insemination had a small POF (ranging 5 to 8 mm) at CIDR removal, and they did not get pregnant. Cows that ovulated within 3 days were classified based on the POF size as follows: 1) small follicles (SF): POF < 10 mm, 2) medium follicles (MF): 10 ≤ POF < 11 mm, and 3) large follicles (LF): POF ≥ 11 mm. There was no difference in conception rates between SF (78.0%), MF (73.5%) and LF (62.2%). Luteolysis during CIDR treatment occurred in all cows in MF and LF groups, however 39.1% in SF showed no luteolysis. In the cows with non-luteolysis in SF, POF size at CIDR removal was smaller than the luteolysis group in SF, MF and LF groups ($P < 0.05$). In Japanese Black cows, P_4 concentrations during estrus synchronization affects fertility by controlling POF size and its ovulation rate.

Key Words: conception rate, estrus synchronization, Japanese Black cows, luteolysis, preovulatory follicle size

Introduction

Estrus synchronization and timed artificial insemination (TAI) are indispensable methods for managing contemporary breeding and for treating reproductive disorders. Therefore, these methods have been extensively studied[25]. The GnRH or estradiol benzoate (EB)-controlled internal

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drug-releasing device (CIDR)-based estrus synchronization and TAI protocols are widely used in Japanese Black cows. Although these protocols improve the pregnancy rate by increasing the submission rate, the possibility of achieving a high conception rate using these protocols is still questionable. The conception rates in Japanese Black cows have been reported to vary from 14% to 73%[3,21,23,37,42].

One of key factors for the efficiency of estrus synchronization and TAI protocols is the induction of the new follicle wave[7]. Since it is known that the emergence of new follicular wave occurs 3 to 5 days after the start of EB-CIDR treatment regardless of the stage of the estrous cycle[12,13,15,34,46], EB-CIDR treatment are used as the popular protocol for estrus synchronization or TAI in Japanese Black cows.

The preovulatory follicle (POF) size at insemination or CIDR removal has been reported to affect the conception rate in the TAI protocol[8,39,41,49]. Previous studies have reported that in an EB-CIDR-based TAI protocol, the POF size at CIDR removal varied, often producing smaller follicles in the cows[8,28,43]. On the other hand, it has not yet been sufficiently elucidated whether small POF occur and whether small POF size affect fertility in Japanese Black cows treated with the EB-CIDR based estrus synchronization protocol.

It has been reported that progesterone (P_4) concentration and frequency of LH pulses are negatively correlated[6,11]. Since LH pulse is a factor that controls follicle growth[19], changes in the P_4 concentration during estrus synchronization treatment will determine the size of the POF. In CIDR-based estrus synchronization, the P_4 concentration reportedly affects follicle development during CIDR treatment, and the growth of POF is suppressed under higher P_4 concentrations[1,13,16,36,46].

To improve the efficiency of EB-CIDR based estrus synchronization protocol in Japanese Black cows, it is necessary to clarify whether POF size affect conception rate and the factors that influence POF size. Therefore, in the present study, the relationship between P_4 concentration, POF size and conception in EB-CIDR based estrus synchronization protocol was investigated.

Materials and Methods

This study was approved by the Animal Experiment Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (Approved number 20-216).

Experimental design

In experiment 1, POF size at CIDR removal and conception rate was examined to clarify the relationship between the POF size and fertility with EB CIDR-based estrus synchronization protocol.

In experiment 2, corpus luteum (CL) size and P_4 concentration at the start of EB-CIDR treatment and CIDR removal were examined to elucidate the effect of P_4 concentration on POF size at CIDR removal with EB-CIDR based estrus synchronization protocol.

The hormonal treatments and examination schedules in experiment 1 and 2 were shown in Figure 1 and 2, respectively.

Animals and treatments

Experiments 1 and 2 were performed on 247 Japanese Black cows raised on 13 commercial farms in Hokkaido from 2011 to 2020. The cows that did not show apparent estrous symptom after calving or did not show estrus despite of non-pregnant after insemination were used in the experiment. The cows were kept in paddocks during the day and stabled at night. Cows were fed 0–4 kg per day of a concentrate diets with free access to hay. There were two patterns of weaning: one approximately 100 d after calving and the other 5 d after calving.

In experiment 1, 182 Japanese Black cows were used. The average age of the cows was 6.2 y (ranging 2.0 to 12.9 y, median age 6.0 y), and

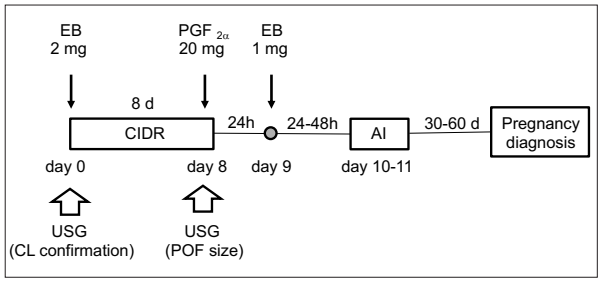

Fig. 1. EB-CIDR based estrus synchronization protocol in experiment 1
Each cow received 2 mg of EB together with CIDR insertion (day 0). The CIDR was inserted for eight days, and 20 mg of $PGF_{2\alpha}$ was injected intramuscularly at CIDR removal (day 8). The cows received 1 mg of EB (day 9) after CIDR removal and were subjected to AI between days 10 and 11. The presence of CL at CIDR insertion and the POF size at CIDR removal were examined using ultrasonography (USG). Pregnancy was diagnosed using USG 30 to 60 d after AI.

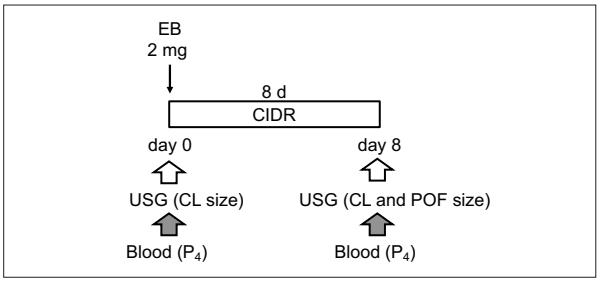

Fig. 2. EB-CIDR based estrus synchronization protocol in experiment 2
Each cow received 2 mg of EB together with CIDR insertion (day 0). The CIDR was inserted for eight days. The CL size at CIDR insertion and removal, the POF size at CIDR removal were examined using ultrasonography (USG). Blood samples for measurement of P_4 concentrations were collected at CIDR insertion and removal.

the average number of postpartum days at the beginning of estrus synchronization protocol was 116 d (34 to 505 d; median day, 89 d).

In experiment 2, 65 Japanese black cows were used. The average age of the cows was 7.5 years (2.0 to 13.9 y, median age 7.2 y), and the average number of postpartum days at the beginning of the estrus synchronization protocol was 108 d (46 to 420 days; median day, 91 d).

Hormonal treatment

In experiments 1 (shown in Fig. 1), after confirming the presence of the CL, the cows were administered 2 mg EB intramuscularly (OVAHORMON®, Asuka Animal Health, Tokyo, Japan) together with the CIDR (CIDR® 1900, Zoetis Japan, Tokyo, Japan) insertion (day 0). The CIDR remained for eight days, and then in only experiment 1, 20 mg of $PGF_{2\alpha}$ (dinoprost tromethamine, Pronalgon®F, Zoetis Japan, Tokyo, Japan) was administered intramuscularly after CIDR removal (day 8). The cows were administered 1 mg of EB intramuscularly (day 9).

In experiments 2 (shown in Fig. 2), after confirming the presence of the CL, the cows were administered 2 mg EB intramuscularly together with the CIDR insertion (day 0). The CIDR remained for eight days.

Artificial insemination and pregnancy diagnosis

Artificial insemination (AI) was performed on days 10–11 in experiment 1 according to the AM-PM rule after the detection of estrus. Visual observation was performed at least twice daily to detect estrus. Cows were recognized to be in estrus when they showed observable standing behavior. If standing estrus was not observed, AI was performed 2 d after EB administration. Pregnancy was diagnosed using an ultrasonography scanner (HONDA HS-101V, Honda Electronics, Aichi, Japan) 30 to 60 d after AI. Pregnancy was considered negative for cows that returned to estrus before diagnosis.

Examination of the ovarian structure using ultrasonography

In all experiments, the ovarian structures were examined using a transrectal ultrasonography (HONDA HS-101V) equipped with a 5 MHz linear array transducer. The follicle identified as having been ovulated (according to the positional relationship between the follicles and CL observed during the protocol) was defined as POF at the time of CIDR removal. Ovulation was confirmed with disappear of a large follicle. Occurrence of ovulation were examined in all cows within 3

Table 1. Association between POF size at CIDR removal and conception rates in Experiment 1 as determined by multivariate logistic regression.

Variable	Category	N[a]	*n*[b]	CR[c]	OR[d]	95% CI[e]	*P*[f]
Independent variable							
POF size at CIDR removal (mm)	SF: < 10	59	46	78.0	1.00	—	—
	MF: 10 to < 11	68	50	73.5	0.74	0.32—1.73	0.486
	LF: 11 ≤	37	23	62.2	0.40	0.15—1.06	0.065
Confounding variables							
Lactation	suckled	90	68	75.6	0.90	0.24—3.40	0.679
	non—suckled	74	51	68.9	1.00	—	—
Postpartum day	< 71	40	29	72.5	2.08	0.41—10.5	0.376
	71 to 87	41	32	78.0	2.81	0.58—13.8	0.201
	88 to 124	42	33	78.6	2.48	0.83—7.39	0.104
	≥ 125	41	25	61.0	1.00	—	—
Age	< 4.5	39	30	76.9	1.67	0.58—4.79	0.344
	4.5 to < 6	42	30	71.4	1.19	0.44—3.22	0.734
	6 to < 7.5	40	28	70.0	0.87	0.33—2.33	0.788
	≥ 7.5	43	31	72.1	1.00	—	—
Day from EB to AI	1	111	78	70.3	0.88	0.39—1.97	0.758
	2	53	41	77.4	1.00	—	—

[a] N: total number of cows.
[b] *n*: number of pregnant cows.
[c] CR: conception rate.
[d] OR: odds ratio.
[e] 95% CI: 95% confidence interval for the odds ratio.
[f] *P*: the probability of the reference category in the variable.

days after insemination. Ovulation checking was maintained until confirmation if ovulation was not confirmed by Day 3. For each of POF and CL, two static images observed at the maximum size were saved, and the image showing the maximum cross-section for POF and the maximum area for CL was used as data. For CL size, the cross-sectional (c-s) area (mm^2) of the CL was calculated using the formula:

CL c-s area (mm^2)=π × (long diameter/2) × (short diameter/2)

Structural luteolysis was defined as a 25% or more decrease in the CL c-s area at CIDR insertion[30].

Blood collection and analysis of P_4 concentrations in the plasma

In experiment 2, blood was collected after CIDR insertion and removed from the coccygeal vein into vacuum tubes containing sodium heparin (VENOJECT® II, VP-H100K, Terumo, Tokyo, Japan). The blood samples were placed in ice-cold water immediately after collection. The samples were transported to the laboratory within 4 h of collection and then centrifuged at 1500 × g for 20 min at 4 °C. The plasma aliquots were stored at -20 °C until further assays were performed. The concentration of P_4 was determined in duplicate using a second-antibody enzyme immunoassay (EIA). All EIA procedures were performed as previously described[33]. The EIA standard curve ranged from 0.05 to 50 ng/mL for P_4. The intra- and inter-assay coefficients of variation (CVs) for P_4 were 7.2 % and 7.0 %, respectively.

Statistical analysis

The statistical analyses in Experiment 1 were performed using SAS version 9.4 (SAS Institute Japan Ltd., Tokyo, Japan). The statistical analysis for Experiment 2 was performed using StatView Version 5.0 (SAS Institute, Cary, NC, USA).

In experiment 1, seven cows were excluded, because they showed a POF size and days

Table 2. The difference in CL size and P_4 concentration, and POF size at CIDR removal between non-luteolysis in SF, luteolysis in SF, and MF and LF groups.

POF size groups at CIDR removal	SF		MF	LF
Rate of change in CL size (%)	< 25 (non-luteolysis)	≥ 25 (luteolysis)	≥ 25 (luteolysis)	≥ 25 (luteolysis)
n	18	28	9	10
CL size at CIDR insert (mm^2)	243 ± 49	297 ± 86^{x}	267 ± 45^{x}	289 ± 122^{x}
CL size at CIDR removal (mm^2)	240 ± 61^{a}	$104 \pm 76^{b,y}$	$35 \pm 40^{b,y}$	$47 \pm 47^{b,y}$
P_4 conc. at CIDR insertion (ng/ml)	4.5 ± 1.3^{X}	5.5 ± 1.7^{X}	5.6 ± 1.9^{X}	5.5 ± 2.1^{X}
P_4 conc. at CIDR removal (ng/ml)	$6.7 \pm 1.7^{a,Y}$	$3.4 \pm 1.8^{b,Y}$	$2.6 \pm 0.6^{b,Y}$	$2.3 \pm 0.6^{b,Y}$
POF size at CIDR removal (mm)	6.0 ± 2.0^{a}	7.7 ± 1.7^{b}	10.4 ± 0.2^{c}	12.0 ± 1.0^{c}

[a, b)] Mean within a row with different superscripts differ at $P < 0.05$
[x, y)] Mean CL size within a column with different superscripts differ at $P < 0.05$.
[X, Y)] Mean P_4 concentration within a column with different superscripts differ at $P < 0.05$.

postpartum that deviated more than three standard deviations from the mean of a POF size (9.9 ± 5.5 mm) at CIDR removal and days postpartum (116 ± 237 day). To identify the factors affecting conception, the data were analyzed using multivariate logistic regression. The dependent variable was defined by the presence or absence of conception. The independent variable was defined by the POF size at CIDR removal. As seasonal factors did not affect the preliminary test, the analysis was performed using the factors causally related to the cows' conditions. The following four confounding variables were used: lactation, days postpartum, age in years, and days from EB to AI. In these cases, the days postpartum and age were categorized based on the quartile points. Values were considered statistically significant at $P < 0.05$.

In experiment 2, to identify the factors affecting the POF size at CIDR removal, the CL size and P_4 concentration at CIDR insertion and removal were examined. Scheffe's multiple comparison tests were used to compare the effect of CL size and P_4 concentration on the POF size at CIDR removal. Student's *t*-tests were used to compare the CL size and P_4 concentration between CIDR insertion and removal in the groups of each POF size. Values were considered statistically significant at $P < 0.05$.

Results

Experiment 1

Eleven cows did not ovulate within 3 days after AI, and they had a small POF size (7.2 ± 1.1 mm, ranging 5 to 8 mm) at CIDR removal. In all of these cows, ovulation was confirmed by 7 days after AI. Within 3 days after AI, 59.3% (16/27) of cows with POF < 9 mm were ovulated, whereas all cows with POF ≥ 9 mm showed ovulation. No cow that did not ovulate within 3 days conceived.

In 164 cows that ovulated within 3 days after AI, the POF size at the time of CIDR removal was organized in quartiles (lower quartile: 9 mm, medium: 10 mm, upper quartile: 10 mm), and three groups were formed based on the quartile point as follows: 1) small follicles (SF): POF < 10 mm, 2) medium follicles (MF): 10 ≤ POF < 11 mm, and 3) large follicles (LF): POF ≥ 11 mm. A comparison of conception rates between SF, MF and LF groups is shown in Table 1. Cows in the SF were selected as the referent group. There was no difference in conception rates among the three POF size groups.

Experiment 2

The difference in CL size and P_4 concentration at CIDR insertion and removal, and POF size at CIDR removal between the SF, MF, and LF groups are shown in Table 2. Although luteolysis occurred in all cows in the MF and LF groups, 39.1%

(18/46) of the cows with SF showed no luteolysis. Therefore, the SF group was further divided into two groups (luteolysis and non-luteolysis). In non-luteolysis group, the CL size was maintained and P_4 concentration was increased from insertion to removal of CIDR. The CL size and P_4 concentration at CIDR removal in the non-luteolysis group in SF was greater ($P < 0.05$) than that of the luteolysis group in SF, MF and LF groups. No differences in the CL size and P_4 concentration at CIDR removal were observed between the luteolysis group in SF, MF and LF groups. In the luteolysis group in SF, MF and LF groups that showed structural luteolysis, P_4 concentration at CIDR removal were significantly decreased compared with at CIDR insertion. In the cows with non-luteolysis in SF, the POF size at CIDR removal was smaller than the luteolysis group in SF, MF and LF groups ($P < 0.05$).

Discussion

Present study using the EB-CIDR based estrus synchronization protocol in Japanese Black cows demonstrated that there was a relationship between POF size at CIDR removal and ovulation rate, which is consistent with previous reports using other breeds of beef cattle[9,13,14,18,44]. In the current study, ovulation was not confirmed within 3 days after AI in about 40% of cows with POF < 9 mm at CIDR removal. The study using the EB-CIDR protocol in beef cows showed that ovulation rates of cows with a follicle size of < 9 mm (63%) at CIDR removal were significantly lower than that of cows with a follicle size of ≥ 9 mm (93% or more)[9]. Beef cows with a small POF of 7 mm at the time of CIDR removal took 90 h from CIDR removal to ovulation, even if EB was administered for ovulation induction on the day after CIDR removal[48]. It has been reported that the expression level of the LH receptor required for ovulation was significantly lower when the POF size at the time of ovulation induction was 8.0 to 9.0 mm than when it was 10.1 to 11.0 mm[44]. Since deviation occurred when the dominant follicle size was 8.9 ± 1.3 mm in Japanese Black cows[2], some of POF < 9 mm at CIDR removal in present study might have insufficient LH receptor expression when ovulation is induced by EB on day nine. Therefore, it was presumed that 11 cows were incapable of ovulating within 3 days after insemination. In all of these 11 cows, delay of ovulation was occurred, and then no conception confirmed. Present study was consistent with the previous study that showed that delay of ovulation of small POF decrease conception rate[40].

In cows ovulated within 3 days after insemination, the POF size had no effect on conception rate. The studies those confirmed ovulation within 2 days after ovulation induction with GnRH showed lower conception rate when small POF ovulated[14,39]. Formation of small CL and low P_4 concentration in serum were suggested as the reason of low conception rate after ovulation of small POF[14,39]. In TAI protocol, a positive correlation between POF size and serum estradiol concentration was observed at the time of ovulation induction by GnRH[5,22]. Furthermore, it has been reported that there is a positive correlation between the estradiol concentration at ovulation induction and the P_4 concentration in the subsequent luteal phase[22]. It has been suggested that estradiol concentrations during the preovulatory period may impact fertility by the effects on the uterine environment through the regulation of estrogen and progesterone receptors[8,38]. It has been reported that in CIDR Co-synch protocol, estradiol cypionate supplementation on 1 day before TAI increased conception rate in beef cows induced to ovulate small dominant follicle[22]. In present study, EB was administrated to induce estrus and ovulation. The reason why the conception rate of SF group was not different from MF and LF groups in present study in contrast to other studies is considered to be the supplementation with estradiol.

Understanding the factors that reduce the POF size in estrus synchronization could

be useful information to improve protocols for clinical practice. Experiment 2 investigated the dynamics of CL size and P_4 during EB-CIDR treatment to elucidate the factor that reduce POF size at CIDR removal. In 39.1% (18/46) of cows with SF, CL size was maintained and P_4 concentration increased. Frequency of LH pulses, that are negatively regulate by P_4[6,11], is a factor that controls follicle growth[19]. Therefore, in the cow of non-luteolysis, the follicle development was suppressed and follicle growth was slow. The occurrence of luteolysis during EB-CIDR treatment observed in the present study was consistent with a previous study[32]. The status of luteolysis during CIDR treatment was different between the SF, MF, and LF groups, and the CL was considered to be functionally luteolytic in all cows with MF and LF. It was shown that in MF and LF, the CL size and P_4 concentration at CIDR removal were significantly lower than in SF, and as a result, the POF size was increased. It has also been reported that cows with a CL at 7 d after the start of CIDR treatment have a smaller POF than cows without CL[34]. In reports with artificially induced luteolysis at the start of CIDR treatment[13,36] or during CIDR treatment[27,29], greater follicle growth was observed when the P_4 concentration decreased due to luteolysis. Taken together, present study clearly demonstrated that changes in the P_4 concentration during treatment will determine the size of the POF.

Interestingly, even in the SF group, more than half of the cows (60.9%) had luteolysis. In the non-luteolysis group in SF, high P_4 concentration was maintained, but in the luteolysis group in SF, the P_4 concentration was decreased. The P_4 concentration at CIDR removal in the luteolysis group in SF was similar to that in MF and LF groups. However, POF size in the luteolysis group in SF was significantly smaller than that in MF and LF groups. Since the CL size at CIDR removal of the luteolysis group in SF was larger than that of MF and LF, it is possible that luteal regression occurred later in the luteolysis group in SF compared with MF and LF. In other words, in the luteolysis group in SF, it is considered that the period, when the P_4 concentration reached a low concentration that promotes follicle growth, was short. However, the exact time of luteolysis during CIDR treatment was not determined in the present study.

There are two possible causes of luteolysis during CIDR treatment. One is spontaneous luteolysis due to the stage of the estrus cycle at the start of EB-CIDR based estrus synchronization protocol[10,20] and the other is that EB-CIDR treatment itself may have induced luteolysis[26,34,35]. Regarding the latter, it has been reported that oxytocin receptor expression in the uterus by the action of estrogen is involved in PGF_{2a} production, causing CL regression[4,17,31,45]. In experiment 2, it is possible that luteolysis was caused by both factors, but since it was not specifically examined, further investigation is required in the future. It has been reported that EB-CIDR treatment in the first half of the estrus cycle is less likely to cause luteolysis during CIDR treatment and will sustain P_4 concentrations[20,34]. Therefore, in the SF group, EB-CIDR treatment may have been started in the first half of the estrus cycle in at least the non-luteolytic cows. To date, the optimal initiation stage of the estrus cycle for estrus synchronization with EB-CIDR treatment has not yet been reported[34]. Therefore, the present study suggests a new possibility that the stage of the estrus cycle at the start of EB-CIDR based estrus synchronization protocol affects fertility. In the present study, the stage of the estrous cycle at the start of EB-CIDR treatment was not defined, therefore, further study is required.

When EB-CIDR based estrus synchronization protocol was used, the delay in the emergence of a new follicular wave might be another factor that caused the POF size to become SF at CIDR removal[15]. However, there are many reports that the emergency of a new follicular wave takes place 3 to 5 days after start of treatment when EB-CIDR treatment are applied under the presence of a functional CL[12,24,34,47]. Therefore,

in the present study in which EB-CIDR based estrus synchronization protocol was started for all cows with functional CL (P_4 concentration of ≥ 1.0 ng/mL), the small POF at CIDR removal were a result of the constant P_4 concentration during CIDR treatment, rather than the delay in the emergence of new follicular waves.

In conclusions, it was found that there was a relationship between POF size at CIDR removal and ovulation rate in the EB-CIDR based estrus synchronization protocol for Japanese Black cows, and delay of ovulation was found in the POF size of < 9 mm. In the cows ovulated without delay, there was no difference in relationship between POF size at CIDR removal and conception rate. It is clearly showed that the decrease in P_4 concentration caused by regression of the CL during treatment increase POF size at CIDR removal. In other word, sustained high P_4 concentration during CIDR treatment reduced POF size at CIDR removal. In EB-CIDR based estrus synchronization protocol in Japanese Black cows, P_4 concentrations during the treatment affects fertility by controlling POF size and its ovulation rate.

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