Original Article

Follicular dynamics during the pre-ovulatory and subsequent first follicular wave stages affect the pregnancy outcome in Japanese Black cows

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Abstract. The diameters of the pre-ovulatory follicles (PF) and the largest follicle during the subsequent first follicular wave (W1LF), and plasma estradiol-17β (E_2) concentrations were monitored on Days 0, 1, 3, 5, and 7 (ovulation = Day 1). Pregnancy was diagnosed on Day 30. Cows were classified into two groups according to the location of the dominant follicle ipsilateral (IG) or contralateral (CG) to the corpus luteum on Day 7. From Days 3 to 7, some follicles that had been determined as the subordinate in the previous examination exceeded the W1LF located in the opposite ovary in terms of the diameter. These were defined as switching (SW), whereas others were defined as non-switching (NSW). The diameter of PF was significantly smaller in pregnant (P) animals than in non-pregnant (NP) animals. The plasma E_2 concentration on Day 0 was significantly higher in P animals than in NP animals and tended to be higher in NSW than in SW. In addition, plasma E_2 concentrations around Days 3 to 7 tended to be higher in P animals of NSW than in NP animals of SW. The conception rates did not differ between IG and CG but were significantly higher in NSW than in SW. In the IG group, the conception rate tended to be higher in NSW than in SW.
Key words: Conception rate, Cow, Estradiol-17β, First-wave dominant follicle, Switching

Establishing pregnancy in cows involves an orchestrated sequence from follicular growth, ovulation, luteal development, and preparation of utero-tubal environments for implantation. Ovarian steroids play an essential role in the regulation of these events. In cows, it has been reported that the uterine progesterone (P_4) content is higher in the ipsilateral to the corpus luteum (CL)-bearing ovary and that P_4 plays a pivotal role in the maintenance of pregnancy [1]. Therefore, luteal function is one of the most crucial factors required for establishing and maintaining pregnancy [2].

The estradiol-17β (E_2) secreted by the largest follicles of the follicular wave negatively regulates the development of subordinate follicles to maintain their dominance [3]. Higher E_2 concentration in peripheral circulation triggers a pre-ovulatory surge of luteinizing hormone (LH) to induce ovulation, when the P_4 level is low [4]. It has been reported that pre-ovulatory follicular morphology and function, in terms of the diameter of ovulatory follicles and plasma E_2 concentration, affect ovulation [5, 6] as well as post-ovulatory luteal development [7], P_4 production [8], and pregnancy outcome [9, 10] in cows.

Follicular dynamics during the first follicular wave (W1) after insemination are also involved in the pregnancy outcome. A previous study reported that the development of the dominant follicle in W1 (W1DF) ipsilateral to the CL was associated with a reduced conception rate when compared to cases contralateral to the CL [11]. Co-localization of W1DF and CL has been reported to result in enhanced blood flow in both W1DF and CL [12]. Notwithstanding these

potentially favorable relationships for conception, the mechanism(s) regarding the negative effect of co-localization of W1DF and CL on the establishment of pregnancy remain unclear.

The complexities of follicular growth during W1 complicate this understanding. The selection of recruited follicles leading to W1DF is not monotonic. Follicles that had been determined as the subordinate in a previous examination might exceed the largest one in terms of diameter; these are defined as switching [3]. In switched cases, the failure of the largest follicle to become the W1DF indicates that it may have been exposed to a singular endocrine situation that would have induced the downturn of the largest follicle and/or the boost of the subordinate [13]. According to previous reports, it is plausible that the local effects of W1DF might vary depending on the follicular deviation classes, including the switching.

However, there is little information regarding the impact of pre- and post-ovulatory follicular dynamics on fertility in cows. To better understand the functional relationships between the periovulatory follicular dynamics and fertility in cows, we investigated the detailed tracing of ovarian structures and plasma steroid profiling during the estrus and subsequent W1 periods. This study aimed to clarify the effects of follicular development patterns from the pre-ovulatory stage to the W1 period on the pregnancy outcomes in beef cows.

Materials and Methods

Animals

The experiments were conducted from April 2018 to June 2019 with 86 multiparous and 13 primiparous (total: 99) Japanese Black cows from seven commercial farms in Fukushima Prefecture, Japan. The mean age was 5.3 ± 3.4 years (mean ± SEM). The parity excluding 24 unknown cows was 2.6 ± 2.4, body condition score (BCS) was 3.84 ± 0.38 and postpartum days of insemination was 76.1 ± 44.3 days. BCS was scored from 1.0 to 5.0 in units of 0.25 as previously established for Japanese Black cattle (http://www.nlbc.go.jp/tottori/

kenkyuuseika/taishaprofairu/index.html). Cows were subjected to the standard management protocols in each farm, fed with hay and concentrate, and allowed permanent access to fresh water and mineralized salt. All cows were confirmed to be free of detectable reproductive disorders prior to the experiment. The experimental design of the present study was approved by the ethical review board of the Fukushima Prefecture Agricultural Mutual Aid Association, and informed consent was obtained from each farm.

Experimental design

The estrus of cows was monitored by observing of standing and mounting behaviors, and mucous discharge from the vulva. All cows were inseminated with frozen semen collected from the proven sires. Ovulation was confirmed within 24 h of insemination by transrectal ultrasonography (Tringa linear; Esaote Medical, Maastricht, Netherlands) using a real-time B-mode scanner with a 7.5 MHz linear array transducer. If a cow did not ovulate a day after insemination, the cow was subjected to re-insemination. The day of confirmed ovulation was defined as Day 1. Ovarian ultrasonography and blood collection were performed on Days 0, 1, 3, 5, and 7. Pregnancy was diagnosed using transrectal ultrasonography on Day 30. A portion of the cows that were confirmed to be pregnant (P) on Day 30 were randomly diagnosed on Day 60 to evaluate pregnancy losses.

Ultrasonography

The diameters of the follicles and CL were measured using B-mode ultrasonography each day. The diameter was defined as the mean length of the major and minor axes on the apparent maximal cross-sectional area of the object. The largest follicle in the ovaries on Day 0 was designated as the pre-ovulatory follicle (PF). On Days 1, 3, 5, and 7, the largest follicle in ovaries was designated as the first-wave largest follicle (W1LF), and the largest follicle on Day 7 was designated as W1DF. Follicular growth was monitored by recording the largest follicle in the left and right ovaries and classified according to the changes in the follicular hierarchy. Some follicles, which had been the second in a previous examination, exceeded W1LF located in the opposite ovary in terms of diameter. These were defined as switching (SW). If the W1LF continued to grow as the largest follicle from Days 3 to 7, it was defined as non-switching (NSW). In the present study, we did not trace the intraovarian turnabout between the W1LF and subordinates from Days 3 to 7 because of difficulties in the exact tracing of the intraovarian follicular lineage. Therefore, in the present study, intraovarian follicular turnabout that possibly occurred from Days 3 to 7 was classified as NSW. The locations of W1DF in ovaries were designated as ipsilateral (IG) or contralateral (CG) groups depending on co-localization in the CL-bearing ovary. Regarding the combination of SW/NSW and IG/CG, cows that were confirmed as SW during the deviation process and eventually became IG were defined as SW_IG. Cows that were confirmed as SW and eventually became CG were defined as SW_CG. Similarly, NSW that eventually became IG were defined as NSW_IG, and NSW that eventually became CG were defined as NSW_CG. In the present study, 99 and 67 cows were used for the examination of IG/CG and SW/NSW, respectively.

Blood sampling

Peripheral blood was collected by jugular venipuncture into 10 ml heparinized vacuum collection tubes (Terumo, Tokyo, Japan), placed on ice immediately after the collection, and centrifuged at $1600 \times g$ for 15 min under refrigerated conditions. The harvested plasma was stored at –30ºC until assayed.

Plasma P_4 assay

Plasma samples collected from 87 cows were used for the P_4 assay. The assay was carried out using a microplate-based enzyme immunoassay, as previously described [14]. The assay sensitivity and intra- and inter-assay coefficients of variation were 37.2 pg/ml, 3.86%, and 9.69%, respectively.

Plasma E_2 assay

Plasma samples collected from 98 cows were used in the E_2 assay. The assay was carried out using a microplate-based enzyme immunoassay following the extraction and defatting of samples. E_2 extraction was performed with a slight modification as in a previous report [15]. Briefly, a plasma sample (2.5 ml each) was extracted by vortex mixing for 3 min with 5 ml of diethyl ether (Wako Pure Chemical, Osaka, Japan). The two phases were separated, and the aqueous layer was snap-frozen by soaking it in a dry ice-cold ethanol bath. The organic layer was decanted into siliconized glass test tubes and evaporated at 37ºC under nitrogen gas. The extraction was performed twice. The dried samples were dissolved in 500 μl of acetonitrile (Wako Pure Chemical) for defatting. Defatting was carried out with a slight modification, as in a previous report [16]. Briefly, 2 ml of *n*-hexane (Wako Pure Chemical) was added to the sample and vigorously mixed for 3 min, after which 1 ml of *n*-hexane was added, and the *n*-hexane layer was removed by aspiration. The defatting procedure was performed three times. The remaining acetonitrile was evaporated at 80ºC under nitrogen gas. The dried samples were dissolved in 125 μl of assay buffer for enzyme immunoassays. The enzyme immunoassay was performed as follows. Fifty μl of the sample was incubated with anti-E_2 antibody (FKA-236E; Cosmo Bio, Tokyo, Japan) and peroxidase-labeled E_2 (FKA-235; Cosmo Bio) in a 96-well microplate precoated with anti-rabbit IgG (Cappel, Cochranville, PA, USA) for 5 h at 15ºC. Furthermore, the plate was washed, and orthophenylenediamine (Wako Pure Chemical), a substrate for peroxidase dissolved in the citrate buffer, was dispensed into the wells, and the plate was further incubated for 45 min in the dark. Finally, 3 M sulfuric acid was added to terminate the color development. The absorbance (A_{492}) of each well was measured using an automated microplate reader (Bio-Rad, Hercules, CA, USA). The assay sensitivity and intra- and inter-assay coefficients of variation were 0.34 pg/ml, 6.00%, and 11.86%, respectively.

Statistical analyses

All data regarding the plasma P_4 and E_2 concentrations, and diameters of W1LF and CL are presented as the mean ± SD. In Figs. 2 and 3, the follicular sizes and steroid concentrations between the groups are presented as box plots. In the box plot, the whiskers represent the maximum and minimum values. Outliers are shown as circles. The box represents the median, first, and third quartiles. The cross mark in the box represents the mean value.

Statistical analyses were performed using the EZR software for R (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [17]. Pregnancy on Day 30 and embryonic loss outcomes (objective variable; qualitative variable) in IG *vs*. CG and SW *vs*. NSW (explanatory variable; qualitative variable) were analyzed using multivariable logistic regression to determine potentially confounding factors of E_2 concentration on Day 0, parity, and BCS (explanatory variable; qualitative variable). In addition, pregnancy outcomes (objective variable; qualitative variable) in PF, which were classified according to mean diameters in units of 0.15 cm (explanatory variable; qualitative variable), were analyzed by logistic

regression. Pregnancy rates among the four groups (NSW_CG vs. NSW_IG vs. SW_CG vs. SW_IG) were analyzed using Fisher's exact test followed by Bonferroni's multiple comparison tests. Plasma steroid concentrations and the mean diameter of PF on Day 0 compared to the P and NP were analyzed using Welch's t-test. The mean diameter of the PF on Day 0 compared to the SW and NSW were analyzed by Welch's t-test. Plasma E_2 concentrations on Day 0 compared to the SW and NSW were analyzed by Student's t-test after logarithmic transformation. Data including follicular and luteal sizes and plasma steroid concentrations measured repeatedly (P vs. NP, IG vs. CG, SW vs. NSW, P-NSW vs. NP-SW) were analyzed by repeated ANOVA. Statistical significance was considered at a probability value of < 0.05. Differences between $0.05 \leq P < 0.10$ were considered a statistical tendency.

Results

Physiological backgrounds

The age, parity, BCS, and postpartum days of insemination of the IG, CG, SW, and NSW groups are shown in Table 1. There was no significant difference between the IG and CG groups and the SW and NSW groups (P > 0.1).

Pregnancy rate, W1DF location, and follicular switching

In the present study, the overall pregnancy rate was 71.7% (71/99). Double ovulation was not observed in any of the animals tested. Therefore, all pregnancies were confirmed as singleton by pregnancy diagnosis on Day 30. The incidence of IG and CG was 51.5% (51/99) and 48.5% (48/99), respectively. There were no significant differences in the distributions of IG and CG. Table 2. shows the odds ratios and 95% confidence intervals from logistic regression that were adjusted for the setting characteristics described above. Pregnancy rates between the IG and CG groups were 66.7% (34/51) and 77.1% (37/48), respectively, indicating no significant difference. Pregnancy rates in the SW and NSW groups were 47.1% (8/17) and 80.0% (40/50), respectively, indicating a significant difference between

the groups (P < 0.05). Pregnancy rates in the NSW_CG, NSW_IG, SW_CG and SW_IG groups were 78.3% (18/23), 81.5% (22/27), 62.5% (5/8) and 33.3% (3/9), respectively, indicating a statistical tendency between the NSW_IG and SW_IG groups ($0.05 \leq P < 0.1$, Fig. 1).

The number of cows reconfirmed for pregnancy was 23/34 in P of IG, 26/37 in P of CG, 5/8 in P of SW, and 21/41 in P of NSW, respectively. The rates of pregnancy loss in the IG and CG groups were 8.7% (2/23) and 11.5% (3/26), respectively, indicating no significant difference between the groups (P > 0.1). In addition, the rates of pregnancy losses in the SW and NSW groups were 20.0% (1/5) and 14.3% (3/21), respectively, indicating no significant difference between the groups (P > 0.1).

Ovarian and hormonal findings on Day 0

Plasma P_4 concentrations on Day 0 did not differ significantly between the P and non-pregnant (NP) animals (P vs. NP, 0.80 ± 0.63 ng/ml vs. 1.08 ± 0.96 ng/ml, Fig. 2-A). However, plasma E_2 concentrations were significantly higher (P < 0.05) in the P animals than in the NP animals (P vs. NP, 9.02 ± 4.49 pg/ml vs. 7.32 ± 3.11 pg/ml, Fig. 2-B). Plasma E_2 concentration on Day 0 tended to be lower in the SW group than in the NSW group (SW vs. NSW, 6.16 ± 1.81 pg/ml vs. 8.13 ± 1.68 pg/ml, $0.05 \leq P < 0.1$, Fig. 2-C). According to the logistic regression analyses, the pregnancy odds significantly decreased by 81.5% (P < 0.05) when switching occurred, and the E_2 concentration on Day 0 tended to be associated with pregnancy (odds ratio: 1.18, $0.05 \leq P < 0.1$, Table 2).

The diameters of PF were significantly smaller in the P animals than in the NP animals (P vs. NP, 1.22 ± 0.16 cm vs. 1.36 ± 0.23 cm, P < 0.05, Fig. 2-D). The mean diameters of PF did not differ between the SW and NSW groups (SW vs. NSW, 1.27 ± 0.22 cm vs. 1.25 ± 0.15 cm, Fig. 2-E). The pregnancy rate was significantly decreased in cows with PF > 1.45 cm in diameter than other cows (P < 0.05, Fig. 2-F).

There was no significant difference in the plasma P_4 and E_2 concentrations, and diameters of the PF between the IG and CG

Table 1. The age, parity, BCS, and postpartum days of insemination of the IG, CG, SW, and NSW groups

	groups	N	Median	Minimum	Maximum	P
Age (years)	IG	51	5	1	11	P > 0.1
	CG	48	4	1	13	
	SW	17	5	1	13	P > 0.1
	NSW	50	4	1	13	
Parity	IG	45	3	0	8	P > 0.1
	CG	45	2	0	10	
	SW	15	3	0	8	P > 0.1
	NSW	46	2	0	10	
BCS	IG	48	4	2.5	4.25	P > 0.1
	CG	47	4	2.75	4.25	
	SW	15	4	3.5	4.25	P > 0.1
	NSW	48	4	3.25	4.25	
Days postpartum (days)	IG	44	69	30	331	P > 0.1
	CG	42	58.5	30	164	
	SW	12	62	30	331	P > 0.1
	NSW	45	62.5	38	111	

There was no significant difference between the IG and CG groups and SW and NSW groups (P > 0.1).

Table 2. Logistic regression analysis for pregnancy outcome on Day 30

	OR	95%CI	P
Location	0.387	0.089–1.680	0.206
Deviation	0.185	0.040–0.858	0.031
BCS	10.800	0.992–117.0	0.051
E_2 on Day 0	1.180	0.987–1.400	0.069
Parity	0.798	0.611–1.040	0.097

Explanatory variable: location, deviation, BCS, E_2 concentration on Day 0, and parity. Location = IG vs. CG. Deviation = SW vs. NSW (** P < 0.05).
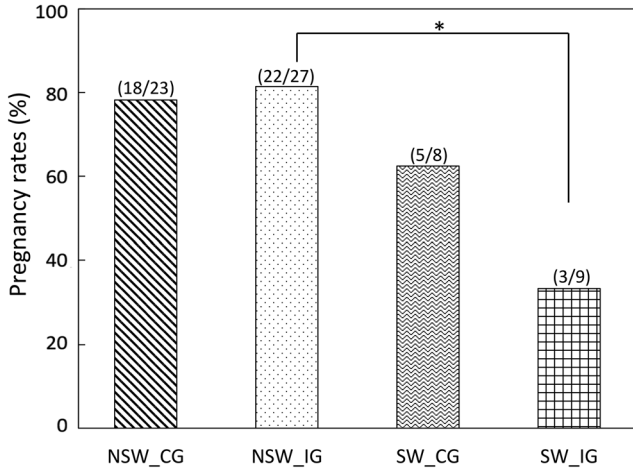
Fig. 1. Pregnancy rates in the NSW_CG (N = 23), NSW_IG (N = 27), SW_CG (N = 8), and SW_IG (N = 9) groups. The numerator and denominator in the parentheses represent the numbers of pregnancies and the total numbers of animals classified into each group, respectively. The asterisk indicates a statistical tendency between the NSW_IG and SW_IG groups (* P < 0.1).

groups (data not shown).

Ovarian and hormonal findings during W1

Following ovulation, changes in the plasma P_4 and E_2 concentrations, and mean diameters of W1DF and CL did not differ between the P and NP animals, IG *vs*. CG, and SW *vs*. NSW (Fig. 3). Plasma E_2 concentrations from Days 3 to 7 compared to among the NP animals of NSW (N = 9), NP animals of SW (N = 8), P animals of NSW (N = 40), and P animals of SW (N = 4) did not differ among the groups (P > 0.1), except between the P animals of NSW and NP animals of SW group indicating statistical tendency between the groups (0.05 ≤ P < 0.1, Fig. 4).

Discussion

The physiological backgrounds, including age, parity, BCS, and postpartum days of insemination, were similar between the IG and CG, and the SW and NSW groups.

In the present study, the distribution of the W1DF location in ovaries ipsilateral or contralateral to the CL was almost the same as that previously reported in dairy cattle [11]. However, in contrast to the previous report, the present study shows no significant difference in the pregnancy rates between the IG (66.7%) and CG (77.1%)
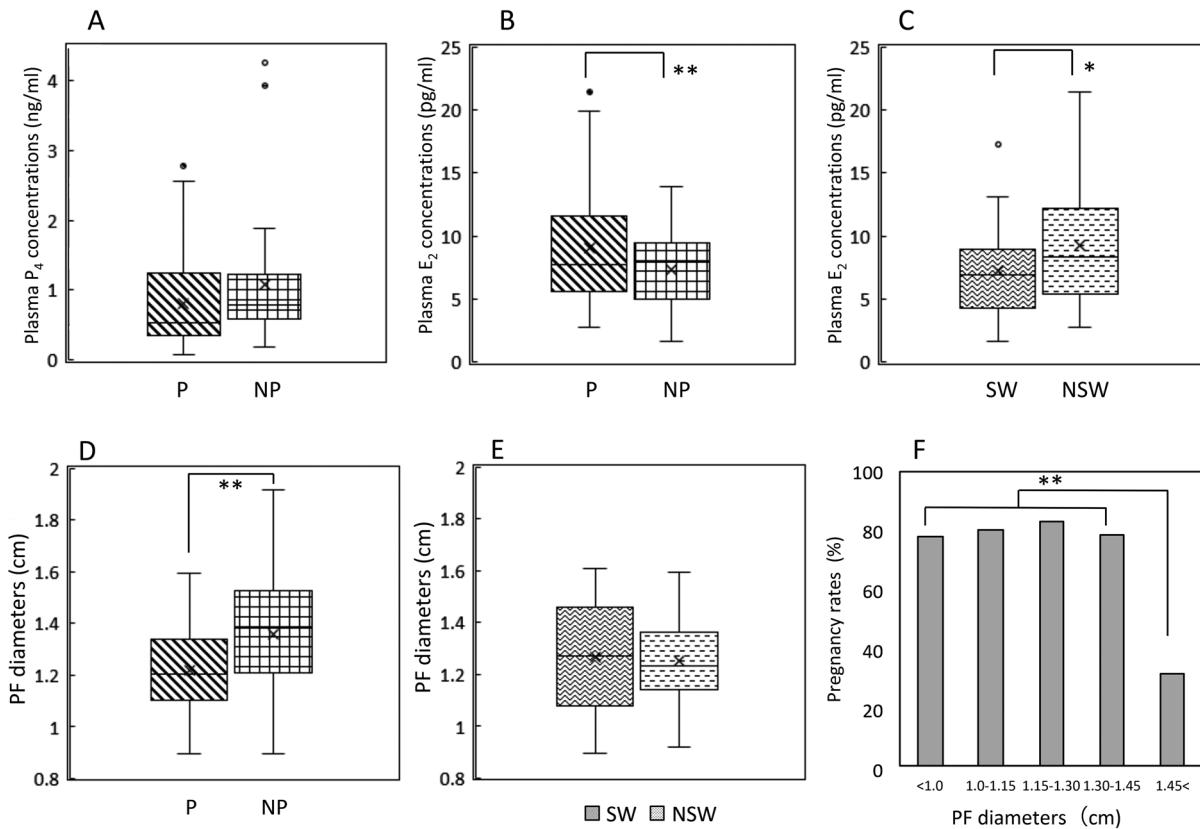

Fig. 2. Plasma P_4 and E_2 concentrations (panels A–C) and mean diameters of PF (panels D–F) on Day 0. A comparison of the plasma P_4 and E_2 concentrations between the P and NP groups are shown in panels A (P: N = 60, NP: N = 27) and B (P: N = 71, NP: N = 28), respectively. Plasma E_2 concentrations compared to the SW (N = 16) and NSW (N = 50) groups are shown in panel C. A comparison of the PF diameters between the P (N = 71) and NP (N = 27) groups (panel D), or between the SW (N = 16) and NSW (N = 50) groups (panel E). In panels A–E, the results are shown as box plots. Panel F shows the effect of PF diameter on the pregnancy rate. In panel F, animals were classified according to the mean diameters of PF in units of 0.15 cm (< 1.0: N = 9, 1.0–1.15: N = 20, 1.15–1.30: N = 30, 1.30–1.45: N = 23, 1.45 <: N = 16). The asterisk and double asterisk denote the statistical tendency and significance between the groups, respectively (* P < 0.1, ** P < 0.05). In panel F, the double asterisk denotes the significant difference between PF >1.45 cm in diameter and others (** P < 0.05).
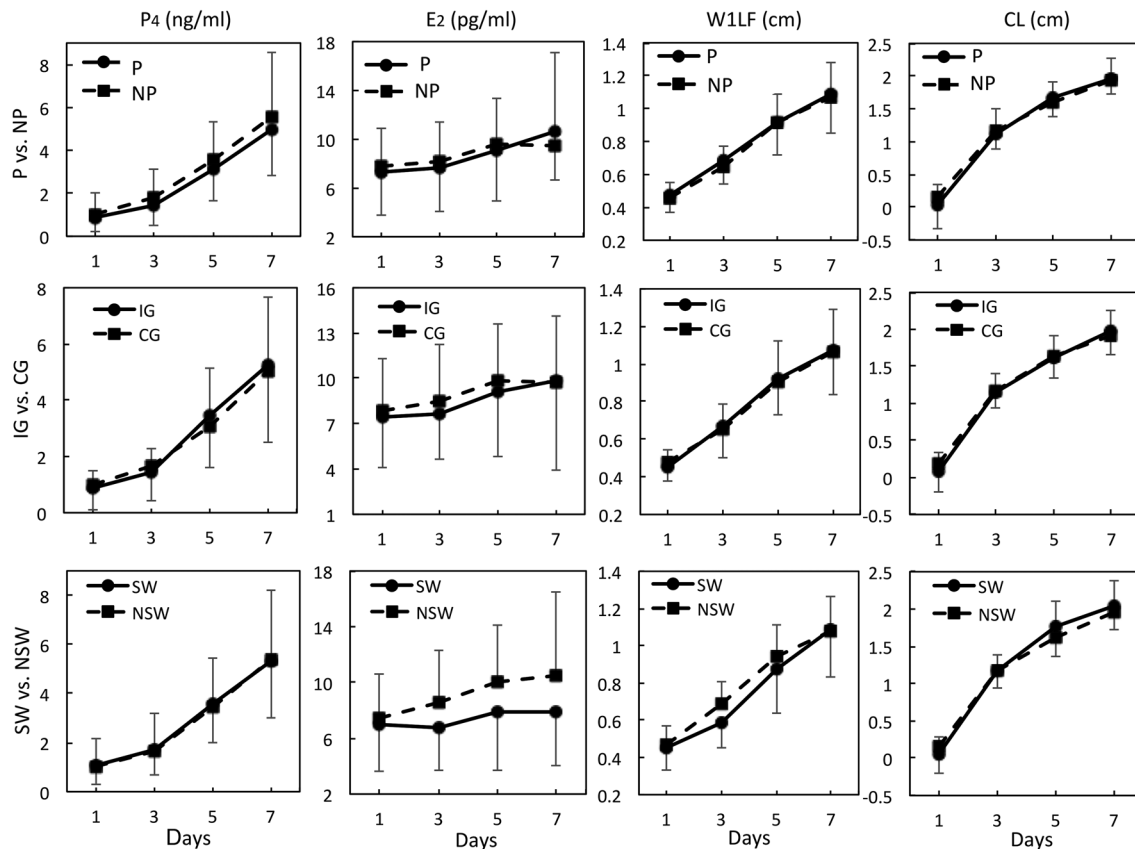
Fig. 3. Plasma P_4 and E_2 concentrations, and the diameters of W1DF and CL from Days 1 to 7. The results are shown in comparison between P animals (P_4: N = 57, E_2: N = 70, W1LF: N = 66, CL: N = 68) and NP animals (P_4: N = 22, E_2: N = 25, W1LF: N = 23, CL: N = 22) (upper), between IG (P_4: N = 42, E_2: N = 49, W1LF: N = 47, CL: N = 45) and CG (P_4: N = 38, E_2: N = 46, W1LF: N = 42, CL: N = 42) (middle), and between SW (P_4: N = 9, E_2: N = 12, W1LF: N = 14, CL: N = 13) and NSW (P_4: N = 39, E_2: N = 49, W1LF: N = 50, CL: N = 48) (lower). The data represent the mean ± standard deviation.
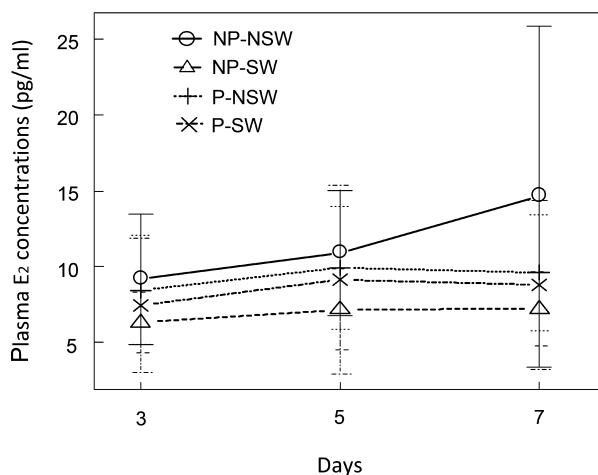

Fig. 4. Plasma E_2 concentrations from Days 3 to 7 in comparing the NP animals of NSW (N = 9), NP animals of SW (N = 8), P animals of NSW (N = 40), and P animals of SW (N = 4). There were no significant differences among the groups (P > 0.1), except between the P animals of the NSW and NP animals of the SW indicating statistical tendency (0.05 ≤ P < 0.1). The data represent the mean ± standard deviation.

groups. Miura et al. [11] reported that different pregnancy rates between the IG and CG groups were not affected by the season, days in milk at insemination, milk yield, BCS, parity, and body weight, suggesting innate body mechanisms in dairy cattle. In the present study using Japanese Black cows, it is noteworthy that the difference in the pregnancy rate between the IG and CG groups (66.7% vs. 77.1%) seems to be less than that reported in a previous study using Holstein Friesian cattle (40.2% vs. 69.3%, respectively) [11], suggesting a greater reduction in the pregnancy rate in the IG group in Holstein Friesian cattle than in Japanese Black cows. Although the detailed mechanism(s) of different pregnancy outcomes between the IG and CG groups remain unclear, it is plausible that the breed is one factor that affects the reduced conception rate in IG animals. It has been reported that high-producing dairy cows have elevated hepatic blood flow owing to increased feed intake, resulting in enhanced steroid clearance [18]. The more prominent reduction in the pregnancy rate of IG animals in Holstein Friesian cattle might be because of the fragility against the detrimental conditions of local utero-tubal-ovarian microenvironments, possibly due to the immoderate feeding for extreme milk yield. There was no significant difference in embryonic loss rates between the IG (8.7%) vs. CG (11.5%) and SW (20.0%) vs. NSW (14.3%). Silke et al. [19] reported that the embryonic loss rates from Days 28 to 84 were 7.2% in dairy cows and 6.1% in heifers. Moreover, in a meta-analysis, Reese et al. [20] reported that the late embryonic mortality rate from Days 32 to

100 was 5.8% (4.8–6.9%) in beef cattle. Embryonic loss can be due to several factors, including genetics, maternal age, progesterone levels, and nutrition [21]. In the present study, the embryonic loss rates of SW and NSW were relatively higher than those reported previously. However, since the physiological backgrounds were similar in both the SW and NSW groups, there was no differences in the embryonic loss rate between SW and NSW.

The results of the present study show that when follicular switching occurred and resulted in the SW_IG, the pregnancy rate tended to decrease compared to the NSW_IG group. In the present study, logistic regression analysis showed that the incidence of follicular switching, the largest follicle replacement by the opposite ovary, was a significant determinant of reduced fertility. A previous report [11] indicated an inferior pregnancy rate in IG animals in dairy cattle without observation of the follicular switching. In the present study, there was no difference in the pregnancy rates among the NSW_CG, NSW_IG, and SW_CG groups, but the rates tended to be lower in the SW_IG group than in the NSW_IG group, suggesting a greater impact of the follicular deviation on the pregnancy outcome than the location of W1DF (Fig. 1). Although, the mechanism(s) regarding the negative effect of co-localization of W1DF and CL on pregnancy outcome remains unclear, our current observation considering the deviation status may provide crucial insight for understanding of the local effect of W1DF. Further research on the impact of intraovarian follicular turnabout is needed.

The results of the present study indicate a decreased pregnancy rate in cows with ovulatory follicles greater than 1.45 cm in diameter (Fig. 2-F). Several contradictory results have been reported regarding the relationship between the size of the PF and pregnancy outcome in dairy and beef cattle. Using gonadotropin-releasing hormone (GnRH)-based timed-AI protocols, Colazo *et al.* [22] reported that the PF diameter was not associated with pregnancy outcome in dairy cows. In contrast, Perry *et al.* [9] reported that GnRH-induced ovulation of dominant follicles not more than 11 mm in diameter resulted in reduced pregnancy outcome and increased pregnancy loss in beef cows. The decrease in fertility following ovulation from smaller follicles was associated with lower concentrations of plasma E_2 on the day of insemination and suboptimal luteal P_4 production, resulting in insufficient plasma P_4 concentrations [9]. In addition, it has been reported that a greater concentration of plasma E_2 on the day of final GnRH injection in the Ovsynch protocol increased the probability of pregnancy in dairy cows [23]. However, in terms of the relationship between the size of PF and pregnancy probability, the above study [23] reported a quadratic relationship between the PF size at final GnRH injection of the Ovsynch protocol and pregnancy probability suggesting the optimal PF size for the best pregnancy outcome. In contrast, the results in the present study indicate no detrimental effects of smaller PF diameters on Day 0 on the pregnancy outcome. Since cows enrolled in the present study had their estrus detected by the observation of specific signs, the physiological maturity of the PF appeared to be sufficient even if the diameter of the PF was small.

Results obtained from the study of the *in vitro* maturation, fertilization, and culture of bovine oocytes indicated that follicular diameter affects both oocyte diameter and developmental competency after fertilization. Arlotto *et al.* [24] reported that larger follicles tend to have larger oocytes, and as the diameter of oocytes increases, so does their developmental potential. In addition, Otoi *et al.* [25] studied the *in vitro* maturation, fertilization, and culture of bovine oocytes/embryos and reported that the rates of cleavage and development to blastocysts increased as the oocyte diameter increased. However, no oocytes (0/7) over 130 μm in diameter developed into blastocysts. The results of the present study imply that PF with an excess diameter has a negative impact on the developmental competency of the embryo in cows.

Plasma E_2 concentration on Day 0 tended to be higher in the NSW group than in the SW group. The production of E_2 by PF is a crucial factor that affects the expression of estrous signs and subsequent luteal progesterone production [26, 27]. It has been reported that E_2 autonomously activates aromatase activity in the granulosa cell layer [28]. It is plausible that higher E_2 levels, derived from the granulosa cell layer of PF on Day 0, prime the estrogenic competency of small follicles that will be recruited for the next follicular wave. Increased secretion of E_2 during the first follicular wave following ovulation drives the negative feedback loop between the ovaries and hypothalamus and results in the reduction of follicle-stimulating hormone (FSH) secretion to below the requirements of subordinate follicles but is sufficient for the requirements of W1LF. After that, the largest follicle continues to grow depending on LH and, in turn, develops into W1DF. To summarize the mechanisms of follicular switching during the first wave, low E_2 levels on Day 0 could result in low E_2 production during the first wave, leading to the failure of depression of FSH secretion.

Although the plasma E_2 concentration on Day 0 was significantly higher in the P group than in the NP group, there was no significant difference between the groups during Days 3 to 7. In the present study, the logistic regression analyses suggest that the follicular switching (odds ratio: 0.185) has a greater impact on the pregnancy outcome than the plasma E_2 concentration on Day 0 (odds ratio: 1.18). Since each P and NP groups included the both SW and NSW, it is plausible that the involvement of NSW in the NP animals and/or SW in the P animals resulted in the similar profiles in plasma E_2 concentrations between the P and NP groups during the Days 3 to 7.

In the present study, we analyzed plasma E_2 concentrations from Days 3 to 7 in the NP animals of NSW, NP animals of SW, P animals of NSW, and P animals of SW to explain the interactions of pregnancy outcome, follicular switching, and plasma E_2 concentrations. However, there were no significant differences among the groups except between the P animal of the NSW and NP animals of the SW groups, indicating statistical tendency (Fig. 4). Jinks *et al.* [8] reported that embryo donor and recipient cows with higher concentrations of serum E_2 on the day of estrus were more likely to yield an embryo and establish pregnancy, respectively. Interestingly, it has been reported that oviductal and endometrial E_2 contents are dozens of times greater than the plasma concentration and are relative to peripheral plasma concentrations, although there is no evidence for E_2 synthesis in both tissues [29, 30]. The roles and mechanisms of the deposition of E_2 in the oviductal and uterine tissues remain unclear. However, the necessity of sequestrated E_2 for the expression of specific functions is inferred. Prior to the study by Mann *et al.* [30], Miller *et al.* [31] compared the effects of higher and lower doses of E_2 treatment on ovariectomized ewes to mimic the pre-ovulatory period. The ewes were subjected to embryo transfer four days after estrus. The results indicated that the ewes that did not receive E_2 treatment were less likely to maintain pregnancy, had less uterine weight, and had a lesser amount of uterine luminal proteins on Day 21 post-estrus, suggesting crucial effects of E_2 on uterine function and embryonic development. Later, Madsen *et al.* [32] also suggested the beneficial effects of pre-ovulatory E_2 treatment on embryo survival and pregnancy establishment, focusing on the uterine receptivity.

Knickerbocker *et al.* [33] and Thatcher *et al.* [34] reported that E_2 administration caused an increase in the plasma concentration of

13,14-dihydro-15-ketoprostagrandin $F_{2\alpha}$ (PGFM), the main metabolite of $PGF_{2\alpha}$ in cattle, suggesting the regulatory effects of E_2 on prostanoid synthesis. In addition, a recent study reported that estradiol increased PGE_2 and $PGF_{2\alpha}$ production in bovine endometrial explants [35]. Since then, it has been reported that PGE_2 causes dilation of the uterine vascular bed in ewes [36, 37], and the luteolytic effect of $PGF_{2\alpha}$ is less potent during the early luteal phase in cattle, E_2 may have a positive impact on the establishment of pregnancy by regulating utero-tubal microenvironments via PG synthesis. In the present study, it is suggested that higher plasma E_2 levels during W1 originating from higher E_2 production of PF facilitate embryonic survival by improving the utero-tubal microenvironment.

In conclusion, results in the present study show that the pregnancy odds were significantly decreased by 81.5% when switching occurred, suggesting a greater impact of the follicular deviation patterns on the pregnancy outcome rather than the location of W1DF. It is also suggested that lower plasma E_2 concentrations on Day 0 could result in the lower E_2 concentrations around Days 3 to 7 leading to the failure to maintain the dominance of the largest follicle.

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