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Original Article

Relationship between ovarian ultrasonographic findings on the seventh post-estrus day and plasma progesterone concentration, nutritional metabolic factors, and pregnancy outcome in dairy cows

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Abstract. To improve the accuracy of ultrasonographic assessment of luteal function, we investigated the relationship between ovarian ultrasonographic findings on Day 7 (Day 1 = ovulation) and plasma progesterone (P_4) concentration, nutritional metabolic factors, and pregnancy outcome. A total of 47 spontaneous estrus events were investigated in 38 lactating Holstein cows (artificial insemination, n = 31; embryo transfer, n = 16). Transrectal ultrasonography was performed on Days 0 and 7 to measure the pre-ovulatory follicle area on Day 0 and the luteal tissue area (LTA), luteal blood flow area (LBF), relative LBF (rLBF) (= LBF/LTA), and dominant follicle area (DFA) on Day 7. Blood samples were collected on Day 7 to measure plasma P_4, insulin-like growth factor-I (IGF-I), insulin, and metabolites. Plasma P_4 concentration was positively correlated with LTA but was not associated with LBF or rLBF. Plasma P_4 concentration was positively correlated with blood glucose and IGF-I and negatively correlated with blood urea nitrogen and free fatty acid, and no significant relationship was found between the ultrasonographic findings of the corpus luteum (CL) and these blood metabolites. Pregnant cows had smaller DFA than non-pregnant cows. In conclusion, LTA measurement can help predict plasma P_4 concentration, but it was difficult to detect variations in plasma P_4 concentration in relation to changes in energy status by evaluating the CL ultrasonographically. A combined assessment of CL and first-wave dominant follicle may be important in evaluating fertility.
Key words: Corpus luteum, Dairy cow, Metabolic hormone, Ovarian ultrasonography, Progesterone

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Fertility in lactating dairy cows has been declining worldwide over the past few decades [1]. One possible cause of the decline in fertility is a decrease in circulating progesterone (P_4) concentrations [2]. P_4 secreted from the corpus luteum (CL) plays an essential role in establishing and maintaining pregnancy [3]. In particular, an increase in P_4 concentration during the first week after estrus has a major effect on conceptus elongation [4, 5], enhances interferon-tau secretion from the embryo [6, 7], and increases pregnancy rates [8, 9]. However, circulating P_4 concentrations are reportedly low in modern high-yield dairy cows due to energy deficiency and increased metabolism of steroid hormones in the liver [1, 2, 10]. Circulating P_4 concentrations are lower in lactating dairy cows than in heifers [11]. Therefore, in lactating dairy cows, accurate evaluation of the P_4 secretory function of the CL and the formation of a CL with greater P_4 secretory function are important for fertility improvement.

Measurement of plasma P_4 concentration is the gold standard for evaluation of luteal function [12, 13, 15]. However, owing to the relatively high cost of the P_4 assay and the time required to obtain results after blood collection, transrectal ultrasonography is typically used in clinical practice to assess luteal function. In ultrasonography, luteal size is commonly measured because it correlates with plasma P_4 concentration [13]. Additionally, measurement of the size of the first-wave dominant follicle (DF) [14] and, more recently, measurement of luteal blood flow using color Doppler ultrasonography have also been performed [15–19]. First-wave DF has been reported to secrete estradiol (E_2) [20] and may negatively affect fertility [14], while luteal blood flow has been reported to be higher in pregnant cows than in non-pregnant cows after artificial insemination (AI) [16] and embryo transfer (ET) [17]. Therefore, these ultrasonographic findings can be used as indicators to select cows with high fertility and improve reproductive management. However, the factors causing variations in these ultrasound findings remain unclear. Results regarding the relationship between luteal blood flow and pregnancy outcomes vary in the literature [18], and the mechanism by which luteal blood flow enhances fertility is not known. To more accurately assess luteal function and fertility using ultrasonography, it is necessary to elucidate the factors that cause variation in these ultrasonographic findings.

Metabolic hormones, such as insulin-like growth factor-I (IGF-I) and insulin, are potential factors that can cause variations in luteal function. In particular, IGF-I is considered a major factor in signaling nutritional status to the hypothalamus–hypophyseal–ovarian axis, which affects follicular development, CL formation, and steroidogenesis in the ovary [10, 21, 22]. Although there are many reports on the relationship between metabolic hormones, follicular development, and the first ovulation in the early postpartum period when a negative energy balance occurs [23–25], there are few reports on the relationship between metabolic hormones and CL formation during the breeding period. Additionally, to the best of our knowledge, no study has examined the relationship between metabolic hormones and luteal blood flow.

In the present study, to determine the cause of variation in these luteal function indices, we investigated the relationship between ovarian ultrasonographic findings and plasma P_4 and metabolic

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hormone concentrations in lactating dairy cows on the seventh day post-estrus. Additionally, the relationship between these indices and pregnancy outcomes was examined.

Materials and Methods

Animals

The present study was conducted at two commercial dairy farms in Yamagata Prefecture, Japan, from October 2019 to June 2021, with no data collected from July to September to avoid the effects of heat stress. A total of 47 estrus events in 38 lactating Holstein-Friesian cows (25 on Farm A and 13 on Farm B) were examined in this study. The cows were kept in tie stalls and fed dry hay and concentrate supplements separately, with *ad libitum* access to water. They were milked twice daily, and the average daily milk yield was approximately 25 kg on both the farms. These cows were clinically healthy and were more than or equal to 60 days postpartum. The average age, parity, and days postpartum of the cows were 5.1 ± 2.4 years (mean ± SD; 4.6 ± 2.0 at Farm A and 5.9 ± 2.8 at Farm B), 2.9 ± 2.0 (2.4 ± 1.7 at Farm A and 3.7 ± 2.3 at Farm B), and 147.0 ± 67.8 days (149.8 ± 61.9 at Farm A and 131.3 ± 75.5 at Farm B), respectively. The body condition score (BCS) of each cow was evaluated on a 5-point scale with increments of 0.25 [26]. The average BCS of the cows was 2.70 ± 0.36 (2.52 ± 0.20 at Farm A and 2.99 ± 0.39 at Farm B). All experimental procedures were approved by the University Committee for the Use and Care of Animals of Tokyo University of Agriculture and Technology (No. R02-97).

Experimental design

In the present study, only cows in spontaneous estrus were examined, and no estrus synchronization procedures were performed. Following visual detection of estrus by the farmers, transrectal ultrasonography was performed to confirm estrus based on the presence of a pre-ovulatory follicle and regressed CL. Ovulation was confirmed 24 h later using transrectal ultrasonography. If a cow did not ovulate, transrectal ultrasonography was performed 24 h later to confirm ovulation. The day of confirmed ovulation was defined as Day 1. Thirty-one cows were artificially inseminated on Day 0, and 16 cows were subjected to transcervical ET on Days 7 or 8. Commercially frozen *in vivo*-fertilized embryos (Zen-noh ET Center, Hokkaido, Japan) or *in vitro*-fertilized embryos (Animal Bio-Technology Center, Livestock Improvement Association of Japan, Tokyo, Japan) were used for ET. The embryos were thawed according to the manufacturer's instructions and transferred into the uterine horn ipsilateral to the ovary bearing the CL. Ovarian ultrasonography was performed on Days 0 and 7 and blood samples were collected on Day 7. Pregnancy was diagnosed by transrectal ultrasonography on Days 40–50 to confirm the fetal heartbeat, and cows in estrus before Day 40 were diagnosed as non-pregnant.

Ovarian ultrasonography

Follicular and CL sizes were evaluated using the B-mode of a portable ultrasound device (MyLabOne VET; Esaote S.p.A, Genoa, Italy) equipped with a 10-MHz linear array probe. The largest follicle on Day 0 was defined as the pre-ovulatory follicle (PF), and the largest follicle on Day 7 as the first-wave DF. If a cow had two CLs due to double ovulation, it was excluded from the study. For each follicle and CL, cross-sectional images with maximum areas were recorded and exported to a personal computer. The PF area (PFA), DF area (DFA), and luteal tissue area (LTA) were measured using an image processing software (Image J; Version 1.50, U.S. National Institutes of Health, Bethesda, MD, USA). When a cavity was present within the CL, the LTA was calculated by subtracting the cavity area from the total CL area.

Luteal blood flow was assessed using the color Doppler mode of the same ultrasound device. The probe was placed at the point of the maximal cross-sectional area of the CL, and images without flash artifacts and with the maximal number of color pixels in the luteal parenchyma were recorded. The recorded images were exported to a personal computer, and the area of color pixels within the CL was measured using Image J as the luteal blood flow area (LBF). Relative luteal blood flow area (rLBF), defined as the proportion of the luteal tissue area occupied by colored pixels indicating blood flow, was calculated by dividing the LBF by the LTA. To minimize the variations in recording, the settings of the color Doppler mode (total gain: 80%, color-flow Doppler mapping frequency: 5.0 MHz, pulse repetition frequency: 0.5 kHz) were fixed and used for all examinations. All ultrasonographic examinations were performed by the same operator.

Blood sampling

Blood samples were collected from the coccygeal vein 4 h after feeding using two types of vacuum tubes coated with sodium fluoride/sodium heparin/EDTA-2Na (Venoject II VP-FH052K; Terumo, Tokyo, Japan) for plasma glucose (Glu) analysis and with only sodium heparin (Venoject II VP-H100K; Terumo) for analysis of plasma hormones and metabolites other than Glu. The samples were immediately placed in iced water after collection and then centrifuged at 1,670 × *g* for 15 min within 1 h of collection. Harvested plasma, except for samples for Glu analysis, was stored at –50°C until further analysis.

Hormone and metabolite assays

Plasma P_4 concentrations were determined using a commercial chemiluminescent enzyme immunoassay kit (Access progesterone; Beckman Coulter, Brea, CA, USA). The assay sensitivity and intra- and inter-assay coefficients of variation were 0.1 ng/ml, 3.4%, and 1.9%, respectively. Blood Glu, urea nitrogen (BUN), total cholesterol (T-Cho), and free fatty acid (FFA) levels were measured using an automatic analyzer (AU-680; Beckman Coulter). Plasma IGF-I concentrations were determined in duplicate using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Quantikine ELISA Human IGF-I Immunoassay; R&D Systems, Minneapolis, MN, USA), with 100% cross-reactivity with bovine IGF-I. The assay sensitivity and intra- and inter-assay coefficients of variation were 0.01 ng/ml, 4.3%, and 5.9%, respectively. Plasma insulin concentrations were measured in duplicate using a commercial ELISA kit (Mercodia Bovine Insulin ELISA; Mercodia, Uppsala, Sweden). The assay sensitivity and intra- and inter-assay coefficients of variation were 0.025 µg/l, 3.8%, and 7.1%, respectively.

Statistical analyses

Statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) [27]. All experimental data are presented as the mean ± SE. After confirming a normal distribution using the Shapiro–Wilk normality test, Pearson's correlation coefficient or Spearman's rank correlation coefficient was calculated to examine the relationships between ovarian ultrasonographic findings and blood parameters. Comparisons of ovarian ultrasonographic findings and blood parameters between pregnant and non-pregnant cows were performed using Student's *t*-test or Mann-Whitney U test. Differences and

correlations with P-values less than 0.05 were considered significant, and those with P-values between 0.05 and 0.1 were considered to indicate tendencies.

Results

Relationships between plasma P_4 concentration and ovarian ultrasonographic findings

The relationship between plasma P_4 concentration (range: 0.85–8.77 ng/ml) and ovarian ultrasonographic findings is shown in Table 1. Plasma P_4 concentration on Day 7 was positively correlated with LTA ($r = 0.47$, $P < 0.01$) (Fig. 1A) but was not associated with LBF, rLBF, or DFA (Fig. 1B–D).

One cow had a plasma P_4 concentration of 0.85 ng/ml, which was below 1 ng/ml, suggesting luteal insufficiency [12, 28]. The LTA, LBF, rLBF, and DFA of this cow were 3.61 cm^2, 1.57 cm^2, 0.435, and 1.45 cm^2, respectively, which were within the respective ranges in the other cows (2.44–7.03 cm^2, 0.75–3.15 cm^2, 0.174–0.762, and 1.22–2.91 cm^2) (Fig. 1A–D).

Relationships between ovarian ultrasonographic findings

The relationships between the ovarian ultrasonographic findings are shown in Table 1. LBF was positively correlated with LTA ($r = 0.33$, $P < 0.05$) and rLBF ($r = 0.82$, $P < 0.01$). LTA was positively correlated with PFA ($r = 0.40$, $P < 0.01$), while rLBF tended to show a negative correlation with PFA ($r = -0.28$, $P = 0.057$).

Relationships of plasma P_4 concentration and ovarian ultrasonographic findings with metabolic parameters

The relationships of plasma P_4 concentration and ovarian ultrasonographic findings with metabolites, IGF-I, and insulin are shown

Table 1. Correlation coefficient between plasma P_4 concentrations (P_4) and ovarian ultrasonographic findings

	PFA	LTA	LBF	rLBF	DFA
P_4	0.09	0.47 **	0.14	−0.12	−0.16
PFA		0.40 **	−0.04	−0.28 #	0.03
LTA			0.33 *	−0.19	−0.11
LBF				0.82 **	−0.11
rLBF					−0.07

PFA, pre-ovulatory follicle area on Day 0; LTA, luteal tissue area on Day 7; LBF, luteal blood flow area on Day 7; rLBF, relative luteal blood flow area on Day 7; DFA, dominant follicle area on Day 7. **, * Denote significant correlation ($P < 0.01$ and 0.05) and # denotes correlation trend ($P < 0.1$).

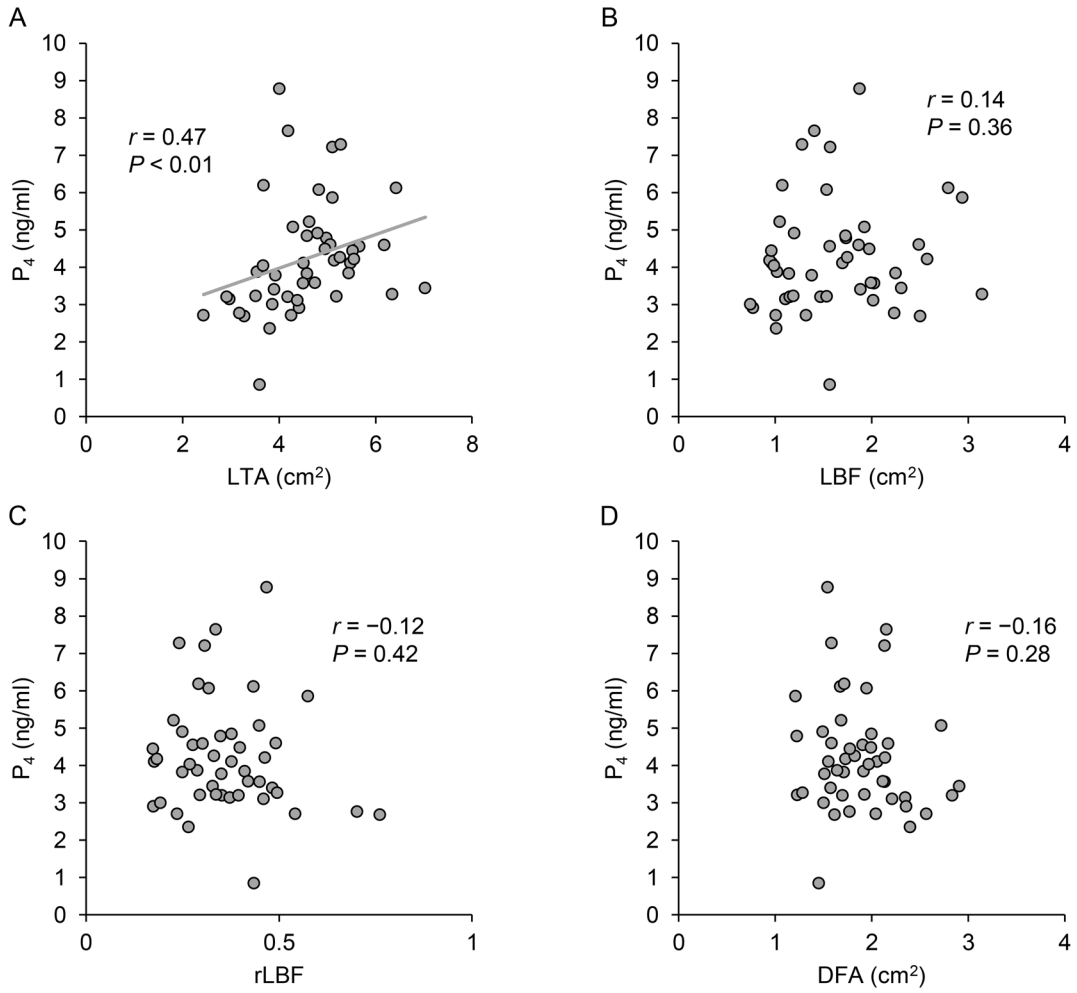

Fig. 1. Relationships of ovarian ultrasonographic findings on Day 7 (LTA, luteal tissue area (A); LBF, luteal blood flow area (B); rLBF, relative luteal blood flow area (C); DFA, dominant follicle area (D)) with plasma progesterone concentrations (P_4) in the 47 estrus events.

in Table 2. Plasma P_4 concentration was positively correlated with Glu (r = 0.45, P < 0.01) (Fig. 2A) and IGF-I (r = 0.29, P < 0.05) (Fig. 2D) and negatively correlated with BUN (r = −0.48, P < 0.01) (Fig. 2B) and FFA (r = −0.35, P < 0.05) (Fig. 2C). No significant relationships were observed between ultrasonographic findings of the CL (LTA, LBF, and rLBF) and blood parameters (Table 2). The DFA tended to be positively correlated with IGF-I (r = 0.26, P = 0.084).

One cow with suspected luteal insufficiency had a plasma IGF-I concentration of 26.8 ng/ml, which was the lowest among the cows examined (Fig. 2D). Glu, BUN, and FFA in this cow were 65 mg/dl, 12.7 mg/dl, and 111 µmol/l, respectively, which were within the respective ranges in the other cows (45– 69 mg/dl, 7.5– 21.5 mg/dl, and 35– 230 µmol/l) (Fig. 2A–C).

Table 2. Correlation coefficients between plasma P_4 concentrations (P_4) and metabolic parameters, and between ovarian ultrasonographic findings and metabolic parameters

	Glu	BUN	T-Cho	FFA	IGF-I	Insulin
P_4	0.45 **	−0.48 **	0.23	−0.35 *	0.29 *	0.06
PFA	0.12	0.08	0.18	−0.06	0.13	−0.11
LTA	0.04	−0.24	−0.08	−0.13	0.05	0.03
LBF	−0.08	−0.13	−0.06	−0.01	0.03	−0.15
rLBF	−0.07	0.04	−0.03	0.04	−0.06	−0.15
DFA	−0.20	0.24	0.13	0.06	0.26 #	−0.15

Glu, glucose; BUN, blood urea nitrogen; T-Cho, total cholesterol; FFA, free fatty acid; IGF-I, insulin-like growth factor-I; PFA, pre-ovulatory follicle area on Day 0; LTA, luteal tissue area on Day 7; LBF, luteal blood flow area on Day 7; rLBF, relative luteal blood flow area on Day 7; DFA, dominant follicle area on Day 7. **, * Denote significant correlation (P < 0.01 and 0.05) and # denotes correlation trend (P < 0.1).

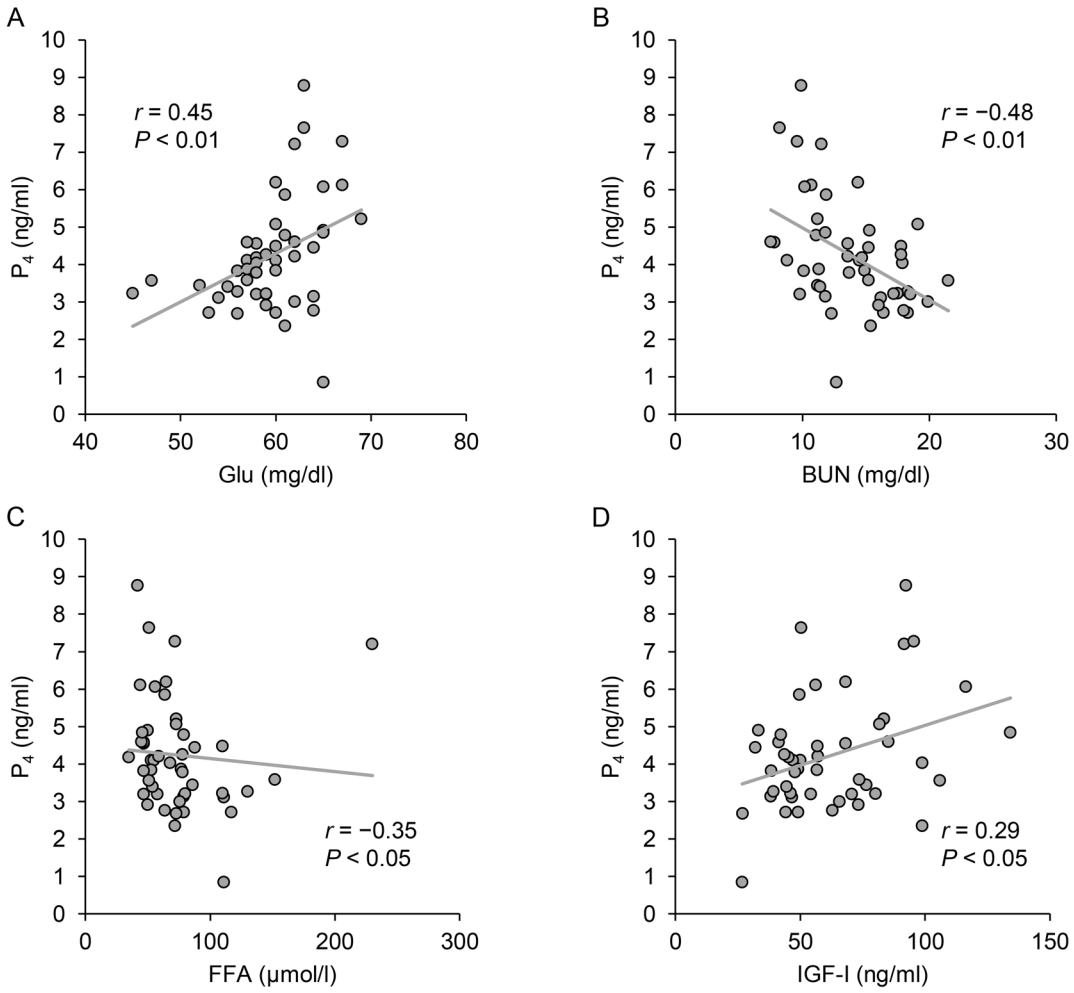

Fig. 2. Relationships of plasma glucose (Glu; A), blood urea nitrogen (BUN; B), free fatty acid (FFA; C), and insulin-like growth factor-I (IGF-I; D) concentrations with plasma progesterone concentrations (P_4) in the 47 estrus events.

Relationship of plasma P_4 and metabolic hormone concentrations and ovarian ultrasonographic findings with pregnancy outcome

The pregnancy rates after AI, ET, and overall were 35.5% (11/31), 56.3% (9/16), and 42.6% (20/47), respectively. No significant differences were observed in plasma P_4 and metabolic hormone concentrations or luteal ultrasonographic findings between pregnant and non-pregnant cows for both AI and ET (Fig. 3A–F). However, DFA tended to be smaller in the AI pregnant cows (1.79 ± 0.10 cm^2) than in the AI non-pregnant cows (2.03 ± 0.09 cm^2; $P = 0.097$), and when the AI and ET results were combined, DFA was significantly smaller in the pregnant cows (1.72 ± 0.07 cm^2) than in the non-pregnant cows (2.01 ± 0.09 cm^2; $P < 0.05$) (Fig. 3G).

Discussion

In the present study, plasma P_4 concentration on Day 7 post-estrus in dairy cows was positively correlated with LTA but not with ultrasonographic findings of luteal blood flow, such as LBF or rLBF. These results were consistent with those of a previous study that showed that the plasma P_4 concentration on Day 9 post-estrus correlated with luteal size but not with luteal blood flow in dairy cows [29]. In contrast, Herzog *et al.* [15] reported that the correlation between plasma P_4 concentration and luteal blood flow during the estrous cycle was stronger than the correlation between plasma P_4 concentration and luteal size. However, they examined the same cows multiple times to determine the relationship between changes in plasma P_4 concentrations and ultrasonographic findings during the estrous cycle. Large individual differences in luteal blood flow have been reported in lactating dairy cows [18]. The present results suggest that luteal size is a more appropriate indicator than luteal blood flow when comparing plasma P_4 concentrations between individuals at one point in the estrous cycle, particularly in the mid-luteal phase.

Similar to the results of a previous study [29], LBF was positively correlated with LTA and rLBF. These findings suggest that LBF is dependent on luteal size and the blood flow per luteal tissue. In the present study, LTA showed a positive correlation with PFA, and rLBF tended to show a negative correlation with PFA. The former result was consistent with that of a previous study that reported that the smaller the ovulatory follicle, the smaller the subsequently formed CL [30]. The latter result may reflect the formation process of the vascular structure of the CL. The development of the vascular structure of the CL commences with the invasion of the vascular endothelial cells of the theca cell layer into the avascular granulosa cell layer after the ovulatory luteinizing hormone (LH) surge, followed by rapid formation of vessels in the CL [31]. The larger the ovulatory

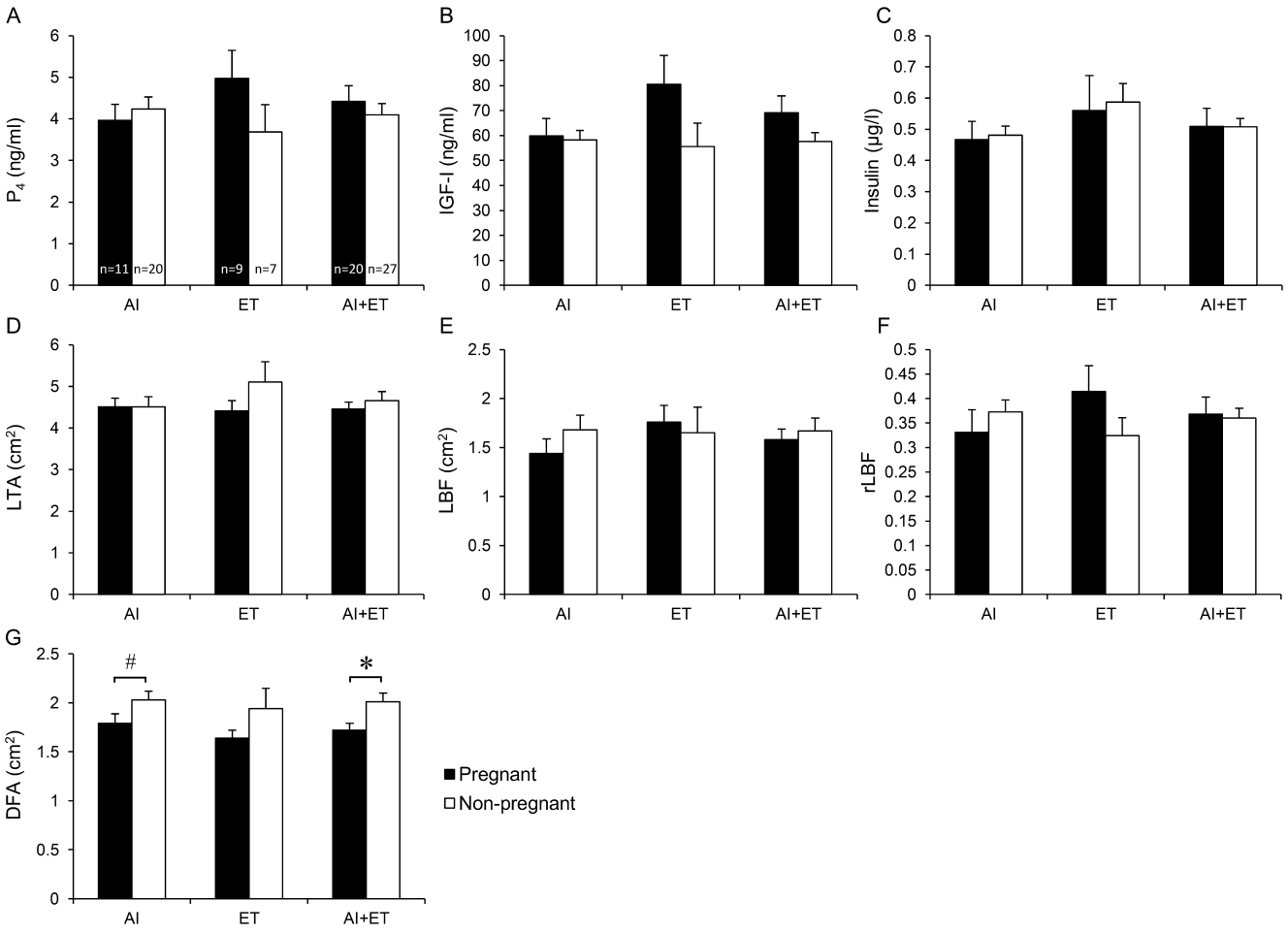

Fig. 3. Comparison of plasma progesterone (P_4; A), insulin-like growth factor-I (IGF-I; B), and insulin (C) concentrations and ovarian ultrasonographic findings on Day 7 (LTA, luteal tissue area (D); LBF, luteal blood flow area (E); rLBF, relative luteal blood flow area (F); DFA, dominant follicle area (G)) between the pregnant (solid column) and non-pregnant (open column) cows. Values are means ± SE. * Denotes significant difference ($P < 0.05$) and $^{\#}$ denotes significant tendency ($P < 0.1$) between the pregnant and non-pregnant cows.

follicle, the smaller the surface area per volume, and consequently the lower the vascular density of the CL, which may have resulted in a negative correlation between rLBF and PFA. These findings suggest that when LBF and rLBF are compared between individuals, they more strongly reflect differences in the vascular structure of the CL than differences in the P_4 secretory capacity of the CL.

In relation to metabolic parameters, plasma P_4 concentration was positively correlated with Glu and IGF-I and negatively correlated with BUN and FFA. High levels of Glu and IGF-I and low levels of FFA were suggestive of a favorable energy status [21], and plasma P_4 concentrations were found to decrease with worsening energy status. BUN has been shown to be a sensitive indicator of the balance between the amount and availability of digestible crude protein (CP) and energy fed to cattle [32]. The BUN of the cows used in this study ranged from 7.5 to 21.5 mg/dl, suggesting a relative excess of digestible CP in some cows. Excess ammonia is generated in the rumen in the presence of excess digestible CP, which is converted to urea in the liver [33]. Because energy is consumed in this process, excess digestible CP has a negative impact on energy status [33]. This could explain the negative correlation between plasma P_4 concentrations and BUN in the present study. In contrast, for ultrasonographic findings, only DFA showed a positive correlation with IGF-I, and no correlation was observed between the ultrasonographic findings of the CL, including LTA, and these blood metabolites. Therefore, it would have been difficult to detect variations in plasma P_4 concentration in relation to energy status by evaluating the CL ultrasonographically in this study. These results suggest that it is important to consider not only ultrasonographic findings but also the metabolic status of the animal for the diagnostic evaluation of plasma P_4 concentration in dairy cows.

One of the cows used in this study had a low plasma P_4 concentration (0.85 ng/ml), suggesting luteal insufficiency [12, 28]. In humans, luteal insufficiency due to reduced luteal blood flow has been reported [34]. However, the LBF and rLBF of this cow were within the range of measurements of the other cows, suggesting that the luteal insufficiency was not caused by blood flow. The plasma concentrations of Glu, BUN, and FFA in this cow were within the respective ranges of the other cows, while the plasma IGF-I level was the lowest among the cows examined. IGF-I has been reported to stimulate P_4 secretion from luteal cells and luteal tissue *in vitro* [21, 35]. As the LTA of this cow was within the range of that of the other cows and no luteal hypoplasia was observed, the cause of luteal insufficiency accompanying inadequate P_4 secretion from the luteal cells may be related to the low level of blood IGF-I concentration.

P_4 concentrations and ultrasonographic findings of the CL did not differ between pregnant and non-pregnant cows in either the AI or ET. In contrast, Hart *et al.* [16] reported that LBF and rLBF on Day 10 post-estrus in pregnant dairy cows that underwent AI were greater than those in non-pregnant cows. Kanazawa *et al.* [17] also reported that LBF on Days 7 and 14 post-estrus in pregnant dairy cows that underwent ET was greater than that in non-pregnant cows. The reason for the discrepancy between these results and the present results is unclear, although one possible reason may be the difference in the experimental setting. In previous reports [16, 17], estrus synchronization procedures were used in some or all cows, which may cause ovulation in physiologically immature follicles in some cows [36]. Based on the results of this study, the degree of ovulatory follicle development may have a major effect on the variability in LBF and rLBF. Therefore, the fluctuation patterns of LBF and rLBF may have differed between cows with spontaneous estrus and those treated with estrus synchronization, and the relationship of LBF and rLBF with pregnancy outcomes may also have been different. In the present study, DFA tended to be smaller in pregnant cows than in non-pregnant cows after AI and was significantly smaller in pregnant cows when AI and ET results were combined. It has been reported that first-wave DF secretes E_2 [20], which may adversely affect fertility under high E_2 conditions [14]. Nishigai *et al.* [14] reported a decreasing trend in conception rate when the blood E_2/P_4 ratio was ≥ 1.0 on Days 6 and 7 post-estrus in beef cows subjected to ET. It is possible that cows with a larger DF had higher plasma E_2 concentrations, which may have negatively affected conception.

Regarding the development of the first-wave DF, Adams *et al.* [37] reported that the maximum diameter of the first-wave DF was smaller in cows treated with P_4 for 5 days from the day of ovulation than in those that were not treated, suggesting that the development of the first-wave DF is controlled by plasma P_4 concentration. However, in the present study, no negative correlation was observed between plasma P_4 concentrations and DFA. In contrast, DFA was positively correlated with IGF-I. IGF-I has been reported to stimulate the proliferation of granulosa cells of follicles [38], suggesting that it also affects the development of first-wave DF. In the present study, IGF-I was also positively correlated with the plasma P_4 concentration. In other words, IGF-I may stimulate both P_4 secretion by the CL and DF development during the luteal phase, which may explain why there was no negative correlation between plasma P_4 concentration and DFA. In relation to fertility, although DFA was significantly higher in non-pregnant cows than in pregnant cows, there were no differences in plasma IGF-I and P_4 concentrations between pregnant and non-pregnant cows. Therefore, fertility may be reduced in cows with a larger first-wave DF relative to the plasma IGF-I and P_4 concentrations.

In conclusion, the results of our study suggest that the plasma P_4 concentration on Day 7 after spontaneous estrus is best reflected by LTA and that LBF and rLBF may reflect the process of luteal angiogenesis more closely than the plasma P_4 concentration. Although the measurement of LTA was useful for predicting plasma P_4 concentrations, it was difficult to evaluate variations in the plasma P_4 concentration due to changes in energy status or plasma IGF-I concentration. The present results also suggest the importance of a combined assessment of the CL and first-wave DF during the luteal phase after spontaneous estrus for the evaluation of fertility in dairy cows. These findings may contribute to improved reproductive management of dairy cows in clinical practice.

Conflict of interests: The authors declare that they have no conflicts of interest.

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