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—Original Article—

Administration of gonadotropin-releasing hormone agonist on Day 5 increases luteal blood flow and improves pregnancy prediction accuracy on Day 14 in recipient Holstein cows

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Abstract. This study assessed the effects of gonadotropin-releasing hormone (GnRH) treatment on Day 5 (Day 0 = estrus) on luteal blood flow and accuracy of pregnancy prediction in recipient cows. On Day 5, 120 lactating Holstein cows were randomly assigned to a control group (n = 63) or GnRH group treated with 100 μg of GnRH agonist (n = 57). On Days 3, 5, 7, and 14, each cow underwent ultrasound examination to measure the blood flow area (BFA) and time-averaged maximum velocity (TAMV) at the spiral arteries at the base of the corpus luteum using color Doppler ultrasonography. Cows with a corpus luteum diameter ≥ 20 mm (n = 120) received embryo transfers on Day 7. The BFA values in the GnRH group were significantly higher than those in the control group on Days 7 and 14. TAMV did not differ between these groups. According to receiver operating characteristic analyses to predict pregnancy, a BFA cutoff of 0.52 cm^2 yielded the highest sensitivity (83.3%) and specificity (90.5%) on Day 7, and BFA and TAMV values of 0.94 cm^2 and 44.93 cm/s, respectively, yielded the highest sensitivity (97.1%) and specificity (100%) on Day 14 in the GnRH group. The areas under the curve for the paired BFA and TAMV in the GnRH group were 0.058 higher than those in the control group (0.996 and 0.938, respectively; $P < 0.05$). In conclusion, GnRH treatment on Day 5 increased the luteal BFA in recipient cows on Days 7 and 14, and improved the accuracy of pregnancy prediction on Day 14.

Key words: Blood flow area, Color Doppler ultrasonography, Corpus luteum, Gonadotropin-releasing hormone, Time-averaged maximum velocity

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Pregnancy diagnosis is an invaluable tool for reproduction management in dairy cows [1]. Estrus is a well-defined period that occurs once every 18–24 days in non-pregnant cows. In general, pregnancy diagnosis is conducted in cows ≥ 35 days after artificial insemination (AI) based on the membrane slip technique by transrectal palpation [2]. Since the 2000s, the development of portable ultrasonographic machines has enabled identification of successfully pregnant cows by scanning the uterus and ovaries to visualize the embryonic vesicle and corpus luteum (CL) as early as 26–28 days post-AI [3].

In Holstein cows, the milk production per cow has increased steadily due to a combination of improved management, better nutrition, and intense genetic selection in the last decade [4]. However, high production causes a longer interval from parturition to first visual estrus [5], weak signs of estrus [6], and a decrease in the duration of estrus [7]. Thus, estrus detection has become more complicated in the Holstein cows. Early pregnancy diagnosis increases reproductive efficiency, especially identifying non-pregnant cows before they return to estrus, thereby enabling careful observations of estrus behavior, detecting estrus, and rebreeding at the subsequent estrus. However, reliable and practical methods have not yet been developed for diagnosis before the estrus cycle.

In addition to the morphological observations of tissues and organs obtained by brightness (B)-mode ultrasonography, color Doppler ultrasonography (CDUS) has provided new information regarding blood flow, and it has been applied in horses and cattle since the 1990s [8, 9]. The mammalian CL necessary for conception has one of the highest blood flow rates of any tissue or organ [10]. The blood vascular bed of the developing bovine CL is supplied by a spiral artery that branches directly from the ovarian artery [11]. After ovulation, blood vessels from the theca interna invade the cavity of the relevant follicle and form a network that supplies the luteal cells. This neovascularization is necessary for the delivery of luteal steroids to the general circulation and for providing the circulating substrate, which is used by the luteal cells for progesterone

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(P_4) biosynthesis [12]. Previous studies indicated that the blood flow area (BFA) and/or blood flow velocity in a CL are closely associated with the plasma P_4 concentrations throughout the estrous cycle, thus advancing CDUS a powerful tool for evaluating the CL function [9, 13]. Therefore, recent studies have employed CDUS in practical applications for pregnancy diagnosis [14–18]. Indeed, the BFA of the CL measured by CDUS during early pregnancy is higher in pregnant than non-pregnant dairy cows at Days 19–21 post-insemination [14–17]. In addition, evaluating the luteal blood flow provides a quick, reliable, and consistent test, especially for early detection in non-pregnant cows at Days 20–21 after AI [16, 17]. However, some studies have suggested that evaluating the luteal BFA alone is not sufficient for early pregnancy diagnosis because of its low specificity and sensitivity [14, 15]. In our previous study, we successfully selected recipient cows with a high probability of conception on Day 7 and early pregnancy prediction on Day 14 using the luteal BFA and time-averaged maximum velocity (TAMV) of the spiral artery [18]. We found that the luteal BFA was significantly higher in pregnant cows than non-pregnant cows on Days 7 and 14, and the TAMV of the spiral artery was also significantly higher in pregnant cows than non-pregnant cows [18]. Moreover, we found that BFA was an appropriate pregnancy predictor on Day 7, and the paired BFA and TAMV were reliable for pregnancy prediction on Day 14 in recipient cows [18].

Gonadotropin-releasing hormone (GnRH), a neuropeptide released from the hypothalamus, has a central role in the reproductive function in mammals [19]. Endogenous GnRH triggers the release and synthesis of follicle stimulating hormone (FSH) and luteinizing hormone (LH) [19]. The secretions of FSH and LH increase acutely after the administration of a GnRH agonist in the same manner as the effect of endogenous GnRH [20–22]. Many angiogenic factors produced by the CL, such as vascular endothelial growth factor A (VEGFA) and basic fibroblast growth factor (FGF2), regulate angiogenesis in the developing CL [23]. In addition, FSH and LH are involved in the regulation of angiogenic factors, and thus in vascular development in the human CL [24, 25]. Therefore, it is highly likely that the administration of a GnRH agonist will increase the vascularity of the CL and affect the accuracy of pregnancy prediction based on luteal BFA and TAMV. However, the effects of GnRH treatment on luteal vascularity have not been analyzed previously.

The current study assessed the effects of GnRH treatment on Day 5 on the luteal blood flow and the accuracy of pregnancy prediction in recipient cows. In particular, we addressed the following questions: 1) whether the changes in the CL morphology and luteal blood flow differed in control and GnRH treatment groups; and 2) whether the accuracy of pregnancy prediction was increased by GnRH treatment.

Materials and Methods

Animals and herd management

The current study was conducted using a commercial Holstein Friesian dairy herd in the Miyagi Prefecture, Japan, with a mean number of 60 lactating cows from December 2013 to January 2016. The cows were housed in free stall facilities, calved all the year round, milked twice daily, and fed complete rations (a mixture of forage and concentrate). The mean daily milk production was (mean ± standard error of the mean [SEM], 28.29 ± 2.42 kg/day). All cows were transferred with embryos recovered from Japanese black beef throughout the year. The present study used 133 cows in total (age, 4.20 ± 0.17 years; 2.48 ± 0.14 parity) during two lactations. The estrus of these cows was checked after calving based on visual observations (mounting and standing behavior) and transrectal palpation. The cows had normal estrous cycles and were ≥ 50 days postpartum (137.58 ± 7.17 days). Body condition scores (BCS) were recorded on Day 0 (estrus) by the same investigator using a scale of 1–5 in increments of 0.25 [26]. The mean BCS of the cows was 3.24 ± 0.04. The experimental design of the present study was approved by the ethical review board of the Miyagi Prefectural Federated Agricultural Mutual Aid Association.

Estrus synchronization

Estrus in cows from the spontaneous (SP) estrus group (n = 71) was detected without synchronization. Cows in the synchronized (SY) estrus group (n = 62) were treated according to the following protocol for fixed-time embryo transfer (ET), as described previously [16]: (1) 2 mg of estradiol benzoate (EB; Kyoritsu Seiyaku, Tokyo, Japan) by intramuscular injection (i.m.) with simultaneous insertion of an intravaginal P_4-releasing device containing 1.9 g of P_4 (CIDR 1900; Zoetis Japan, Tokyo, Japan) on Day –12; (2) prostaglandin-$F_2\alpha$ analogue (500 μg, i.m.; cloprostenol; Estrumate, Nagase Medicals, Hyogo, Japan) and CIDR 1900 removal on Day –2; and (3) a second i.m. administration of EB (1 mg) on Day –1. The day when the recipient cow was observed to be in standing estrus was treated as Day 0.

GnRH treatments

On Day 5, animals were randomly assigned to the control group (cows untreated with GnRH agonist, n = 63) or GnRH group (cows treated with GnRH agonist, n = 57). Animals in the GnRH group received an injection with 100 μg of GnRH agonist (Fertirelin acetate; Conceral; Nagase Pharm, Osaka, Japan) after ultrasound examination and blood sampling for the plasma P_4 assay, as described in Fig. 1.

Embryo transfer

Previous studies have reported that the functional CL diameter in Holstein cows on Day 7 ranges between 19–28 mm [27, 28]. In addition, Murakami *et al*. [29] suggested that the pregnancy rate in recipients with a CL diameter ≥ 20 mm was higher than that in recipients with smaller CL diameters. Therefore, 120 cows with CL diameters ≥ 20 mm on Day 7 were subjected to transcervical ET by the same investigator (Fig. 1). ET was conducted on Day 7 after ultrasound examination and blood sampling for the plasma P_4 assay, as described in Fig. 1. Fresh embryos (n = 48) or embryos thawed from a frozen state (frozen-thawed; n = 72), recovered from superovulated Japanese Black cows, were transferred into the uterine horn ipsilateral to the CL-bearing ovary using a flexible transfer catheter (Mo-No.4, Misawa Medical Industry, Kasama, Japan). All embryos used in the present study were quality grade 1 and developmental stage 4 according to the International Embryo Transfer Society (http://www.iets.org/) classifications.

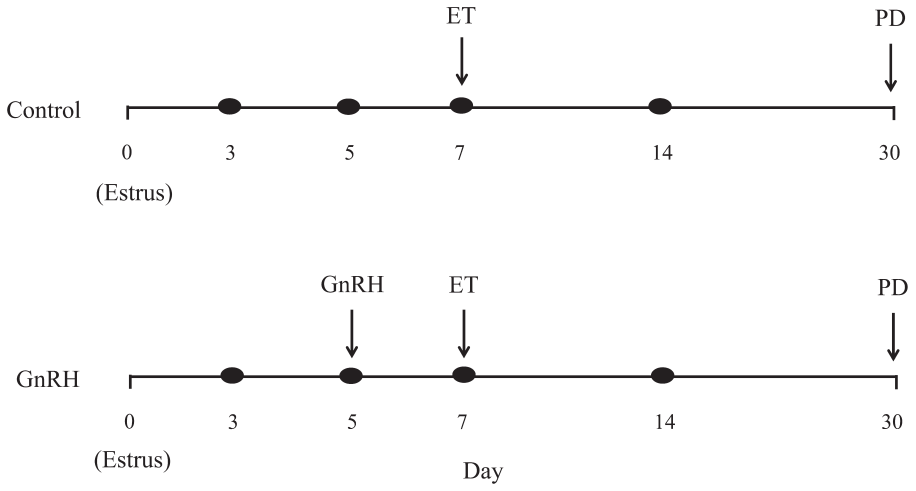

Fig. 1. Schematic views of the treatments, ultrasound examinations, and sampling schedules. The day when the recipient cow was observed in standing estrus was treated as Day 0. Ultrasound examination of ovaries and blood sampling (●) were conducted on Days 3, 5, 7, and 14. Cows in the gonadotropin-releasing hormone (GnRH) group received 100 μg injection (intramuscular) of the GnRH agonist on Day 5 after ultrasound examination and blood sampling. Embryo transfer (ET) and pregnancy diagnosis (PD) were conducted on Day 7 after ultrasound examination and blood sampling and Day 30, respectively.

Ultrasound examination using CDUS

Transrectal ultrasound scanning using CDUS was performed on each cow before blood sampling on Days 3, 5, 7, and 14 (Fig. 1). On Day 5, the GnRH group was scanned before GnRH treatment (Fig. 1). The morphologies of the original CL and largest follicle (LF) were evaluated using a B-mode portable ultrasound machine equipped with a 7.5-MHz linear array transducer (MyLab five; Esaote SpA). The areas of the CL and LF were measured at the maximum diameter. In addition, the morphology of an accessory CL was evaluated in the GnRH group on Day 14. The BFAs of the original and accessory CL were evaluated using the color-flow Doppler mapping mode at the maximum diameter. The ultrasound imaging settings (B-mode frequency: 7.5 MHz; total gain: 70%; color-flow Doppler mapping frequency: 6.6 MHz; pulse repetition frequency: 1.4 KHz) were standardized and remained constant in all examinations. The original and accessory CLs, luteal cavity, CL tissue, LF areas, and BFA were calculated according to the method described by Kanazawa *et al*. [18]. Blood velocity of the spiral artery was measured at the CL base where spiral artery enters the CL. To identify the spiral artery, pulse Doppler probe was oriented according to the longitudinal axis of the ovary (from the mesovarian to proper ligament poles). Subsequently, the probe was set to visualize the spiral artery at the CL base. Since the spiral artery spins, the blood flow images at the CL base were relatively easily captured. Blood flow velocity waveforms were obtained and recorded during three cardiac cycles to determine TAMV by placing the Doppler sample volume over the maximum color intensity of the spiral artery at the base of the CL and switching on the pulsed Doppler mode. Generally, no correction is made for the interrogation angle because the interrogation angle for small vessels cannot be determined [30]. In the current study, the operator confirmed the interrogation angle of the spiral artery and sought the Doppler signals with the highest achievable amplitude as reported in the previous studies [31–33]. Therefore, TAMV could be measured at an appropriate interrogation angle (ranging from 20 to 60 degrees) in the current study. In addition, we measured TAMV three times and used the average of those as TAMV values. Furthermore, the LF location on Day 7 in the control group and the accessory CL location on Day 14 in the ovary relative to the original CL (ipsilateral or contralateral) were observed. Pregnancy diagnoses were performed on Day 30 using a portable ultrasound system equipped with a 7.5-MHz rectal transducer (Tringa V linear; Esaote SpA, Genoa, Italy) to measure the fetal heartbeat.

Collection of blood sample and plasma P_4 assay

Blood samples were collected by coccygeal venipuncture into a vacutainer tube containing sodium heparin (TERUMO, Tokyo, Japan) during each ultrasound examination (Days 3, 5, 7, and 14; Fig. 1). On Day 5, the blood samples were collected before GnRH treatment in the GnRH group (Fig. 1), placed on ice immediately after collection, and centrifuged at 3,000 × *g* for 15 min within 30 min of collection. The harvested plasma was stored at −80°C until the assay. Plasma P_4 concentrations were measured by enzyme immunoassay analysis, as described by Kanazawa *et al*. [18].

Statistical analysis

All data were expressed as the mean ± SEM. Statistical analyses of the data were performed using SPSS 13 (SPSS Japan, Japan). Fisher's exact test was used to compare the pregnancy rates in the control and GnRH groups, the SP and SY estrus groups, and with the fresh and frozen-thawed embryos. Means were compared among four groups (control pregnant, control non-pregnant, GnRH pregnant, and GnRH non-pregnant) using *t*-tests with Bonferroni's multiple comparisons correction. A probability (P) value < 0.05 was considered significant. In addition, we performed receiver operating characteristic (ROC) analyses by focusing on the three traditionally used predictors (original CL area, CL tissue area, and

Table 1. Effects of estrus type on fertility rates

	Total	Spontaneous estrus (SP)	Synchronized estrus (SY)	*P*-value
Number	133	71	62	
Selected number of recipients [a]	120	65	55	
Selection rate (%) [b]	90.2 (120/133)	91.6 (65/71)	88.7 (55/62)	0.77
Pregnancy rate (%) [c]	59.2 (71/120)	58.5 (38/65)	60.0 (33/55)	1.00

Synchronized = P_4 implant (1.9 g of P_4) plus 2 mg of estradiol benzoate (Day –12), implant removal plus 500 μg of prostaglandin-$F_2\alpha$ analogue intramuscular (Day –2), and 1 mg of estradiol benzoate intramuscular (Day –1). [a] Number of recipients selected based on corpus luteum diameter ≥ 20 mm on Day 7. [b] Transferred relative to estrus detected rate. [c] Pregnant relative to transferred rate. The selection rate and pregnancy rate did not differ between the SP (91.6% and 58.5%) and SY estrus groups (88.7% and 60.0%; P > 0.10).

Table 2. Details of animals in the four groups categorized based on their pregnancy diagnosis on Day 30 [a]

	Total	Control		GnRH		*P*-value
		Pregnant	Non-pregnant	Pregnant	Non-pregnant	
Number	120	63		57		
		35	28	36	21	
Age	4.20 ± 0.17	4.16 ± 0.25		4.25 ± 0.22		0.80
		4.37 ± 0.35	3.89 ± 0.34	4.08 ± 0.25	4.52 ± 0.40	
Parity	2.48 ± 0.14	2.51 ± 0.21		2.46 ± 0.17		0.85
		2.63 ± 0.28	2.36 ± 0.31	2.31 ± 0.19	2.71 ± 0.32	
Days after calving (days)	137.58 ± 7.17	127.06 ± 10.14		149.19 ± 9.89		0.13
		141.20 ± 15.69	109.39 ± 10.76	138.75 ± 12.59	167.10 ± 15.21	
BCS	3.24 ± 0.04	3.21 ± 0.05		3.28 ± 0.06		0.40
		3.25 ± 0.08	3.16 ± 0.06	3.22 ± 0.07	3.38 ± 0.09	
Fresh embryo	48	27		21		
		15	12	11	10	
Frozen embryo	72	36		36		
		20	16	25	11	

[a] All data represent the mean ± standard error of the mean. There were no differences between the control and GnRH group and among four groups (P > 0.10). The pregnancy rates with fresh and frozen-thawed embryos did not differ (P > 0.10). GnRH, gonadotropin-releasing hormone; BCS, body condition score.

P_4 concentrations) and two new predictors (BFA and TAMV) on Days 7 and 14 to identify the optimal cutoff value for predicting pregnancy in the GnRH group. We evaluated every candidate cutoff value to determine the optimal cutoff value based on the geometric distance with 100% sensitivity and specificity [34]. The optimal cutoff value was determined from the data point that minimized the distance. We also evaluated the difference between the two ROC curves obtained from the two groups (control and GnRH groups) using the unpaired DeLong's test [35, 36]. We applied one-tailed statistical tests based on the assumption that GnRH administration increased the predictability of pregnancy.

Results

Pregnancy rates and recipient status

The effects of estrus types, SP, and SY on fertility are summarized in Table 1. The selection rate, which was the proportion of recipients selected based on corpus luteum diameter ≥ 20 mm on Day 7 to estrus detected cows (SP; 91.6% *vs.* SY; 88.7%), and pregnancy rate, which was the proportion of pregnant cows to transferred cows (SP; 58.5% *vs.* SY; 60.0%) did not differ between the SP and SY estrus groups (P > 0.10; Table 1). In addition, there were no differences in BFA, TAMV, plasma P_4 concentrations, CL area, CL tissue area, and LF area throughout the experimental period (Supplementary Fig. 1: online only). When ETs were conducted on Day 7 in the Holstein recipient cows, the pregnancy rates with fresh (54.2%, 26/48) and frozen-thawed embryos (62.5%, 45/72) did not differ significantly (P > 0.10; Table 2). Thus, the data obtained from different estrus types and embryo treatments were pooled and analyzed together.

Moreover, Table 2 summarizes the results obtained for the control (n = 63) and GnRH (n = 57) groups, and for the further subdivided four groups; control pregnant (n = 35), control non-pregnant (n = 28), GnRH pregnant (n = 36), and GnRH non-pregnant (n = 21) groups. The age, parity, days after calving, and BCS did not differ among all groups (P > 0.10; Table 2). The pregnancy rates in the control and GnRH groups were 55.6% (35/63) and 63.2% (36/57), respectively, and did not differ significantly (P > 0.10; Table 2).

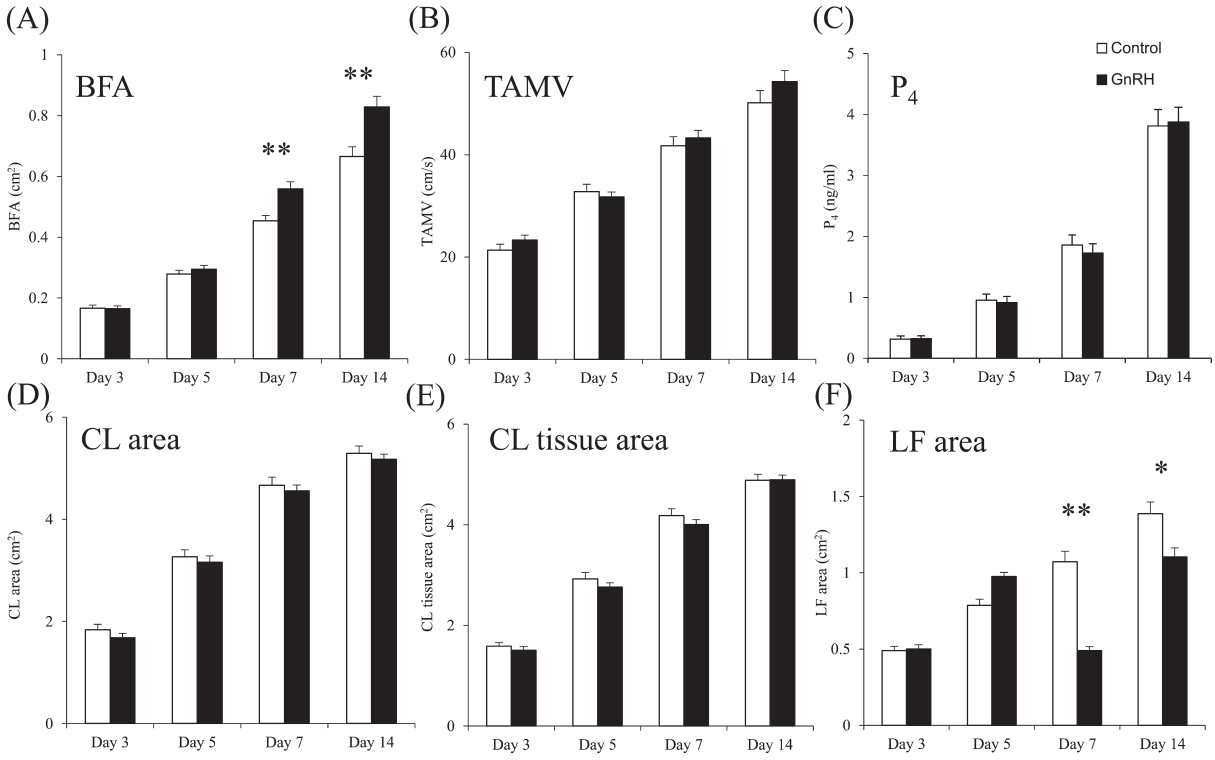

Fig. 2. Changes in the blood flow area (BFA) in the corpus luteum (A), time-averaged maximum velocity (TAMV) of the spiral artery (B), plasma progesterone (P_4) concentrations (C), corpus luteum (CL) area (D), CL tissue area (E), and largest follicle (LF) area (F). White and black bars represent the control group (n = 63) and gonadotropin-releasing hormone (GnRH) group (n = 57), respectively. Cows in the GnRH group received 100 μg intramuscular injection of the GnRH agonist on Day 5 after ultrasound examinations and blood sampling. Embryo transfers were conducted on Day 7 after ultrasound examinations and blood sampling. Data points represent the mean ± standard error of the mean for each day. Significant differences between the two groups identified by *t*-tests with Bonferroni's multiple comparisons correction are marked by asterisks: * $P < 0.05$; ** $P < 0.01$.

Positive effects of a GnRH agonist on luteal blood flow in the original CL

In the control and GnRH groups, the BFA of the CL and TAMV increased gradually throughout the experimental period (Figs. 2A and B). On Days 7 and 14, the luteal BFA was higher in the GnRH group compared with the control group (Day 7: 0.58 ± 0.05 cm^2 *vs.* 0.47 ± 0.02 cm^2 and Day 14: 0.86 ± 0.06 cm^2 *vs.* 0.68 ± 0.03 cm^2; $P < 0.01$; Fig. 2A). The changes in TAMV did not differ ($P > 0.05$) between the control and GnRH groups throughout the experimental period (Fig. 2B). The plasma P_4 concentrations, entire CL areas, and CL tissue areas in the control and GnRH groups increased gradually during the experimental period, but did not differ significantly among groups ($P > 0.05$; Figs. 2C–E). These findings are in agreement with the results of a previous study, which showed that the weight of the original CL after GnRH treatment on Day 5 (Day 0 = estrus) did not differ compared with those from non-treated cows [37].

The LF area was lower in the GnRH group compared with the control group (Fig. 2F) on both Day 7 (0.49 ± 0.03 cm^2 *vs.* 1.07 ± 0.07 cm^2; $P < 0.01$) and Day 14 (1.10 ± 0.06 cm^2 *vs.* 1.39 ± 0.08 cm^2; $P < 0.05$).

Differences in the blood flow (BFA and TAMV) in the original CL between pregnant and non-pregnant cows

The luteal BFA in pregnant cows was higher than that in non-pregnant cows on Days 5 (Fig. 3B), 7 (Fig. 4), and 14 (Fig. 5), regardless of the GnRH treatment ($P < 0.05$). The BFA in the GnRH pregnant group was higher than those in the GnRH non-pregnant group on Days 7 (Fig. 4) and 14 ($P < 0.05$; Fig. 5). In addition, the BFA in the GnRH pregnant group was higher than that in the control pregnant group on Days 7 (Fig. 4) and 14 ($P < 0.05$; Fig. 5). The BFA on Day 3 did not differ between the non-pregnant and pregnant cows (Fig. 3A). The TAMV in the GnRH pregnant group was higher than that in the GnRH non-pregnant group on Day 7 ($P < 0.05$; Fig. 6A), and the TAMV in pregnant cows was higher than that in non-pregnant cows on Day 14, regardless of the GnRH treatment ($P < 0.01$; Fig. 6B). The TAMV on Days 3 and 5 did not differ between the non-pregnant and pregnant cows (Figs. 3C and D). In addition, the CL area, CL tissue area, and plasma P_4 concentrations did not differ among the four groups throughout the experimental period (Supplementary Fig. 2: online only).

Prediction of pregnancy

In the ROC analyses, the closer the plot was to the upper left

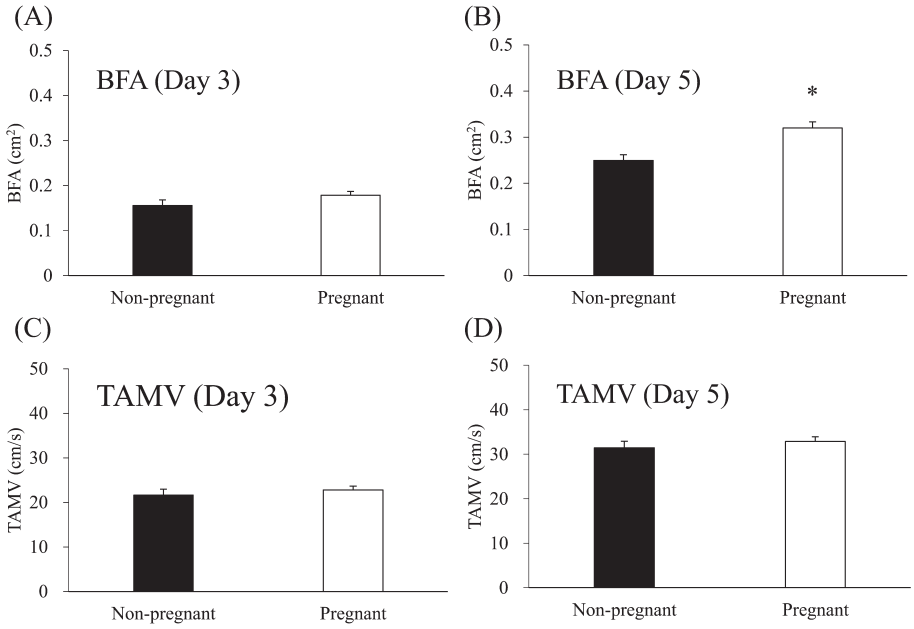

Fig. 3. Blood flow area (BFA) in the corpus luteum (A, Day 3; B, Day 5) and the time-averaged maximum velocity (TAMV) of the spiral artery (C, Day 3; D, Day 5) in the pregnant and non-pregnant groups. Black and white bars represent the non-pregnant group (n = 49) and pregnant group (n = 71), respectively. Data points represent the mean ± standard error of the mean for each group. Significant differences between the two groups identified by *t*-tests are marked by asterisks: * $P < 0.05$.

corner, the higher the overall accuracy of the test [38]. Therefore, the ROC analyses of the current study indicated that BFA provided the most appropriate prediction of pregnancy compared with the other four single predictors (TAMV, CL area, CL tissue area, and P_4 concentrations) on Days 7 and 14 (Supplementary Fig. 3: online only). In the GnRH group, evaluations based on the distance from the ideal point determined the best BFA cutoff value as 0.52 cm^2 for the prediction on Day 7 (sensitivity = 83.3% and specificity = 90.5%; Table 3) and 0.77 cm^2 for that on Day 14 (sensitivity = 86.1% and specificity = 85.7%; Table 4). On Day 14, the most appropriate predictor of pregnancy was the paired BFA and TAMV in the GnRH group (Supplementary Fig. 3D). The best cutoff value at a pregnancy probability of 58.3%, obtained by substituting the date of a pregnant sample with BFA 0.94 cm^2 and TAMV 44.93 cm/s into the best-fit logistic regression equation: *pregnancy probability = 1/{1 + exp [32.7354 − 16.6777(BFA) – 0.4061(TAMV)]}*, yielded the best tradeoff of sensitivity (97.1%) versus specificity (100%) on Day 14 (Table 4). The ROC curves for BFA in the GnRH group were drawn at the upper left compared with those in the control group on Day 7 (Fig. 7A). The area under the curve (AUC) for the BFA in the GnRH group was numerically 0.084 higher than that in the control group (0.891 and 0.807, respectively, P = 0.126; Table 5). On Day 14, the ROC curves for the paired BFA and TAMV in the GnRH group were drawn at the upper left compared with those in the control group (Fig. 7B). The AUCs for the paired BFA and TAMV in the GnRH group were numerically 0.058 higher than those in the control group (0.996 and 0.938, respectively, $P < 0.05$; Table 5).

In addition, we examined whether relative BFA values (BFA per CL area on Days 5, 7, and 14; BFA per CL tissue area on Days 5, 7, and 14; BFA on Day 7 per BFA on Day 5; BFA on Day 14 per BFA on Day 5; and BFA on Day 14 per BFA on Day 7) could serve as better predictors than the exact BFA values. Our results indicated that all these relative values provided less accurate prediction than the exact BFA on Day 7 or Day 14 (data not shown).

Characteristics of LF and accessory CL

Miura *et al*. [39] reported that development of the first-wave (on Days 5–9) dominant follicle (DF) in the ovary ipsilateral to the CL after AI was associated with reduced conception rates in both lactating cows and heifers. Therefore, we thought that luteal BFA and pregnancy might be affected by the LF location. We analyzed the effects of the LF location on Day 7 on the morphology and vascularity of the CL, plasma P_4 concentrations, and pregnancy rates in the control group and summarized the results in Supplementary Table 1 (online only). The LF location (ipsilateral *vs.* contralateral to the original CL) on Day 7 did not affect these evaluated factors except the LF area, which was significantly higher in the ovary ipsilateral to the original CL than in the LF contralateral to the original CL ($P < 0.05$; Supplementary Table 1).

In the GnRH group, the incidence of an accessory CL on Day 14 was 96.5% (55/57; Supplementary Table 2: online only). The area, tissue area, and BFA of the accessory CL did not differ between the GnRH pregnant and non-pregnant groups (Supplementary Table 2). In addition, we analyzed the effects of the accessory CL location (ipsilateral or contralateral to the original CL) on the original CL to rule out the possibility that the accessory CL location would affect the morphology and vascularity of the original CL. Our results suggested the accessory CL location did not affect pregnancy rate,

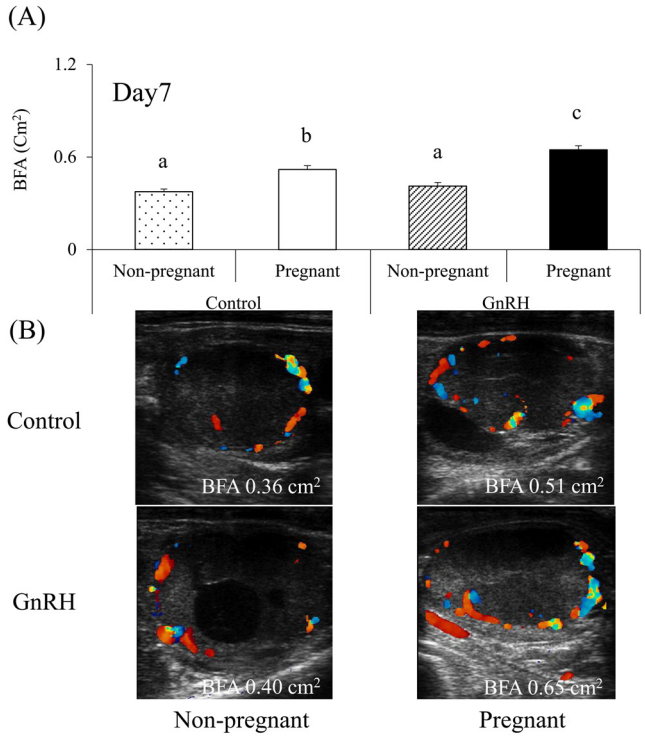

Fig. 4. Blood flow area (BFA) in the corpus luteum (CL) in the control (non-pregnant "dotted column" and pregnant "white column") and gonadotropin-releasing hormone (GnRH) (non-pregnant "slanted-line column" and pregnant "black column") groups (A), and representative images for each group on Day 7 (B). BFA comprises the sum of the colored areas in the CL in each image (B). Embryo transfers were conducted on Day 7 after ultrasound examinations and blood sampling. Data points represent the mean ± standard error of the mean for each group. Letters ($P < 0.05$) indicate significant differences determined by *t*-tests with Bonferroni's multiple comparisons correction.

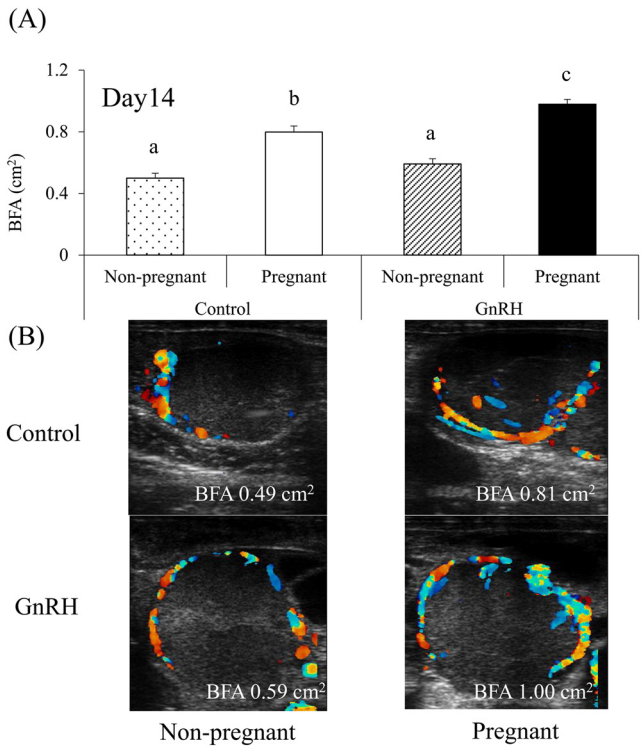

Fig. 5. Blood flow area (BFA) in the corpus luteum (CL) in the control (non-pregnant "dotted column" and pregnant "white column") and gonadotropin-releasing hormone (GnRH) (non-pregnant "slanted-line column" and pregnant "black column") groups (A), and representative images for each group on Day 14 (B). BFA comprises the sum of the colored areas in the CL in each image (B). Data points represent the mean ± standard error of the mean (SEM) for each group. Different letters on the bars ($P < 0.05$) indicate significant differences determined by *t*-tests with Bonferroni's multiple comparisons correction.

the original CL area, tissue area, BFA/TAMV, and plasma P_4 concentrations (Supplementary Table 3: online only). In contrast, the accessory CL location exerted positive effects on its tissue area and BFA (Supplementary Table 3), and the values of BFA and tissue area ipsilateral to the original CL were significantly higher than those of the contralateral accessory CL ($P < 0.01$ and $P < 0.05$, respectively; Supplementary Table 3).

From the results mentioned above, it is thought that there was no indirect influence such as the accessory CL locations on the original CL area, CL tissue area, BFA/TAMV, and plasma P_4 concentrations, regardless of the GnRH administration in recipient cows.

Discussion

Previous studies have reported that the luteal BFA was clearly higher in pregnant than non-pregnant cows during Days 15–21 after AI [15–17] and on Day 19 in recipient cows [14]. However, evaluating the luteal BFA alone was considered insufficient to obtain an early pregnancy diagnosis due to either low specificity and sensitivity [14] or substantial variation among cows [15]. The results of the present study show clearly that the luteal BFA and TAMV were good indicators for use in pregnancy prediction and pregnancy diagnosis on Day 14 in recipient cows. We consider that this success in terms of much earlier pregnancy prediction and diagnosis is attributable to three critical points: (1) using only Holstein Friesian dairy cows; (2) selection on Day 7 based on the CL diameter (≥ 20 mm); and (3) using the paired BFA and TAMV measured by CDUS instead of BFA alone. To refine this new technique for pregnancy prediction and diagnosis further, we attempted to use GnRH, which might increase the blood flow (BFA and TAMV) indirectly, in recipient Holstein cows.

We evaluated the effects of the GnRH treatment (Day 5) on the morphology and blood flow in the original CL as well as the plasma P_4 concentration, where we particularly focused on the luteal BFA and TAMV before (Days 3, 5, and 7) and after (Day 14) ET. This was based on our previous study, which clearly showed that the luteal BFA and TAMV are good indicators for use in pregnancy prediction on Day 7 before ET and pregnancy diagnosis on Day 14 [18]. In the present study, on Days 7 and 14, the GnRH treatment

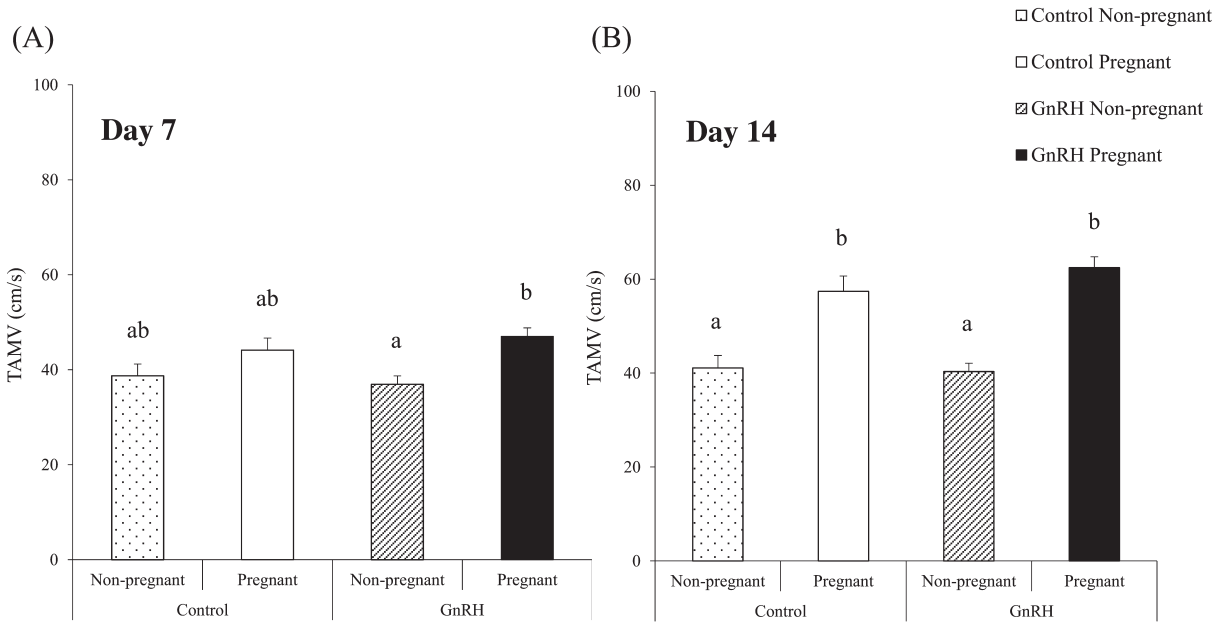

Fig. 6. Time-averaged maximum velocity (TAMV) in the spiral artery in the control (non-pregnant "dotted column" and pregnant "white column") and gonadotropin-releasing hormone (GnRH) (non-pregnant "slanted-line column" and pregnant "black column") groups on Days 7 (A) and 14 (B). Embryo transfers were conducted on Day 7 after ultrasound examinations and blood sampling. Data points represent the mean ± standard error of the mean for each group. Different letters on the bars ($P < 0.05$) indicate significant differences determined by *t*-tests with Bonferroni's multiple comparisons correction.

Table 3. Summary of the ROC analyses of five independent variables in the GnRH group on Day 7

	GnRH		
	Cutoff value	Sensitivity	Specificity
BFA (cm^2)	0.52	0.833	0.905
TAMV (cm/sec)	43.10	0.639	0.810
CL area (cm^2)	4.45	0.500	0.524
CL tissue area (cm^2)	4.19	0.389	0.667
P_4 concentrations (ng/ml)	1.60	0.438	0.667

ROC, receiver operating characteristic; GnRH, gonadotropin-releasing hormone; BFA, blood flow area; TAMV, time-averaged mean velocity; CL, corpus luteum; P_4, progesterone. Evaluations based on the distance from the ideal point determined best BFA cutoff value as 0.52 cm^2 for the prediction on Day 7 (sensitivity = 83.3% and specificity = 90.5%).

Table 4. Summary of the ROC analyses of five independent variables in the GnRH group on Day 14

	GnRH		
	Cutoff value	Sensitivity	Specificity
BFA (cm^2)	0.77	0.861	0.857
TAMV (cm/sec)	50.73	0.861	0.905
BFA and TAMV	0.583[a]	0.971	1.000
CL area (cm^2)	5.20	0.472	0.571
CL tissue area (cm^2)	4.72	0.583	0.476
P_4 concentrations (ng/ml)	3.48	0.515	0.611

ROC, receiver operating characteristic; GnRH, gonadotropin-releasing hormone; BFA, blood flow area; TAMV, time-averaged mean velocity; CL, corpus luteum; P_4, progesterone. [a] Cutoff value at a pregnancy probability of 58.3%, BFA 0.94 cm^2, and TAMV 44.93 cm/sec. The most appropriate predictor of pregnancy was the paired BFA and TAMV in the GnRH group. The best cutoff value at a pregnancy probability of 58.3% (BFA 0.94 cm^2 and TAMV 44.93 cm/sec) yielded the best tradeoff of sensitivity (97.1%) and specificity (100%) on Day 14.

led to increases in the luteal BFA when we compared the GnRH and control groups, regardless of whether the recipient cows were pregnant or not (Fig. 2A). In addition, the luteal BFA was higher in the GnRH pregnant group than controls (pregnant and non-pregnant) and GnRH non-pregnant groups on Days 7 and 14 (Figs. 4 and 5). However, the GnRH treatment did not cause any changes in the TAMV when we compared the GnRH and control groups throughout the experimental period (Fig. 2B). Moreover, the TAMV values of the GnRH and control pregnant groups were comparable on Days 7 and 14 (Fig. 6). These results indicate that the GnRH treatment mainly increases the luteal BFA in pregnant cows and that the changes in BFA were not synchronized completely with those in TAMV. We suggest that TAMV did not increase markedly because dilation of the spiral artery also occurs as the blood supply to the CL increases.

We have previously reported that BFA was an appropriate pregnancy predictor on Day 7, where a BFA cutoff value ≥ 0.43 cm^2 yielded the best sensitivity (79.4%) and specificity (75.0%) [18]. The ROC curve, which is defined as a plot of the test sensitivity as the y coordinate *versus* its 1-specificity or false positive rate as the x coordinate, is used widely and it is an effective method for

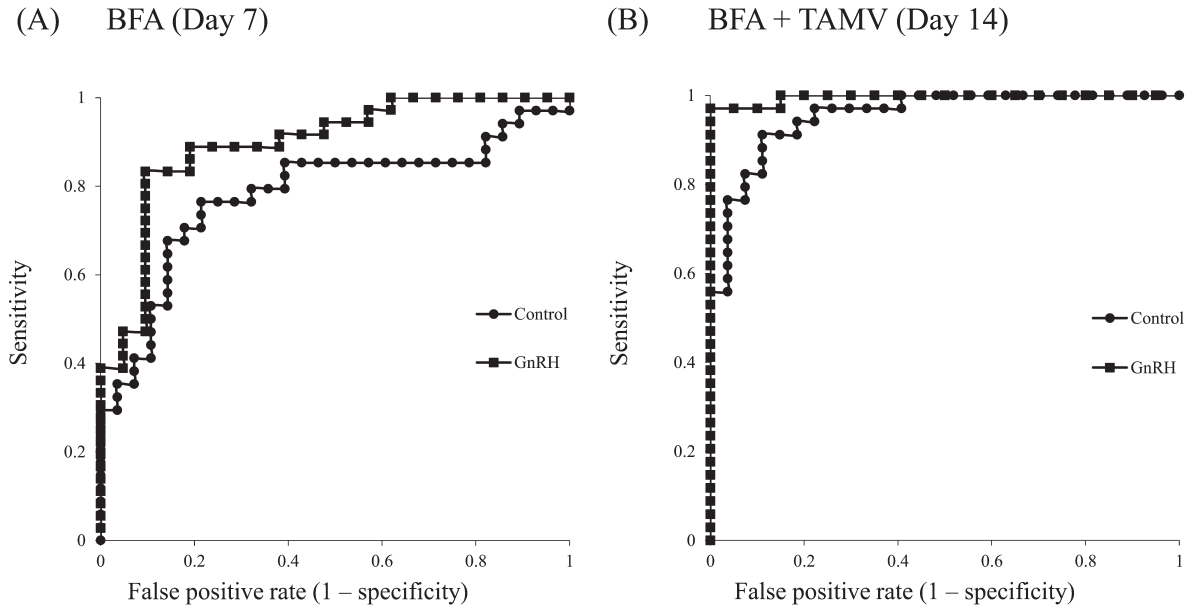

Fig. 7. Receiver operating characteristic (ROC) curves for the blood flow area (BFA) in the corpus luteum on Day 7 (A) and the paired BFA and time-averaged maximum velocity (TAMV) of the spiral artery (B) on Day 14. Embryo transfers were conducted on Day 7 after ultrasound examinations and blood sampling. The horizontal and vertical axes represent the false positive rate (1 – specificity) and sensitivity, respectively; thus, the left upper corner is the ideal point for 100% sensitivity and 100% specificity.

Table 5. Summary of the AUC analyses

	AUC		P-value for unpaired DeLong's test (one-sided)
	Control	GnRH	
ROC for BFA (Day 7)	0.807	0.891	P = 0.126
ROC for BFA and TAMV (Day 14)	0.938	0.996	*P = 0.0273

AUC, area under curve; ROC, receiver operating characteristic; GnRH, gonadotropin-releasing hormone; BFA, blood flow area; TAMV, time-averaged mean velocity. * Significantly different at 5% level. The AUC of the BFA was numerically higher (0.084) than that of the control group (0.891 and 0.807, respectively; P = 0.126). The AUCs of the paired BFA and TAMV in the GnRH group were numerically higher (0.058) than those of the control group (0.996 and 0.938, respectively; P < 0.05).

evaluating the performance of diagnostic tests [40]. Therefore, we performed ROC analyses to determine the cutoff value and found that BFA was also the most appropriate predictor on Day 7 in the GnRH group (Supplementary Fig. 3B). A BFA cutoff value ≥ 0.52 cm^2 yielded the best sensitivity and specificity among all the cutoff values for any data obtained on Day 7 in the GnRH group (Table 3), thereby indicating that GnRH treatment increased the accuracy of pregnancy prediction (sensitivity = 3.9% and specificity = 15.5%) on Day 7 compared with our previous study [18]. However, when we evaluated the difference between the two ROC curves obtained from the control and GnRH groups using the unpaired DeLong's test [35, 36], no differences were observed in the AUC for BFA between the GnRH and control groups (Table 5). Based on these results, we suggest that the GnRH agonist treatment on Day 5 does not significantly improve the accuracy of pregnancy prediction using the luteal BFA as an indicator on Day 7.

Our previous study showed that the paired BFA and TAMV were the most appropriate predictors on Day 14, and the best cutoff value at a pregnancy probability of 60.0% (obtained by substituting the data for a pregnant sample with BFA = 0.63 cm^2 and TAMV = 50.6 cm/sec in the regression equation) yielded a better tradeoff of sensitivity (85.3%) and specificity (91.7%), thereby indicating that the paired BFA and TAMV was reliable for pregnancy prediction on Day14 [18]. According to the ROC analyses, we found that the paired BFA and TAMV was also the most appropriate predictor on Day 14 in the GnRH group (Supplementary Fig. 3D). The best cutoff value at a pregnancy probability of 58.3% (obtained by substituting the data for a pregnant sample with BFA = 0.94 cm^2 and TAMV = 44.93 cm/sec in the regression equation) yielded a better tradeoff of sensitivity and specificity (Table 4), thereby indicating that the GnRH agonist treatment increased the pregnancy prediction accuracy (sensitivity = 11.8% and specificity = 8.3%) on Day 14 compared with the results obtained in our previous study [18]. In addition, the AUC values for the BFA and TAMV in the GnRH group were significantly higher than those in the control group (Table 5). These results indicate that the GnRH treatment on Day 5 significantly improved the accuracy of pregnancy prediction on Day 14. This improvement in the pregnancy prediction was obviously caused by the increased differences in the BFA values for the GnRH non-pregnant and GnRH pregnant groups on Day 14, because the GnRH treatment on Day 5 specifically increased BFA in the GnRH pregnant group, but not in the GnRH non-pregnant group (Fig. 5).

GnRH triggers the release and synthesis of LH and FSH [19].

Several distinct cell types are distributed in the bovine CL, such as small and large luteal cells, vascular endothelial cells, and pericytes [41]. More than 50% of the cells in the mature CL comprise vascular endothelial cells and adequate angiogenesis is essential for normal luteal development [41]. The CL produces many angiogenic factors such as VEGFA and FGF2, which are the major factors that regulate angiogenesis in the developing CL [23]. FSH and LH are involved in the regulation of angiogenic factors, and thus in human CL vascular development [24, 25]. Fátima *et al*. [42] indicated that FSH increases the protein expression levels of VEGFA, FGF2, and their receptors in bovine luteal and granulosa cells. Thus, the increased BFA in the GnRH group on Days 7 and 14 may have been caused by elevated FSH or LH levels promoting vascular development after GnRH treatment. Beindorff *et al*. [43] reported that the luteal BFA increased at 1 h after the administration of human chorionic gonadotropin in non-lactating and non-bred cows on Day 7 (Day 1 = ovulation), but they subsequently returned to the baseline levels. Human chorionic gonadotropin acts in a similar manner to LH by binding LH receptors on small luteal cells in sheep [44]. However, the administration of the GnRH agonist acutely increases FSH as well as LH, which are involved in the regulation of angiogenic factors. Therefore, different changes in the luteal BFA were thought to be observed in the current study. Interestingly, on Day 5, the luteal BFA in the pregnant cows was already higher than that in the non-pregnant cows (Fig. 3B). This result indicates that the CL of the cows that will be pregnant has high blood flow compared with the non-pregnant cows before GnRH treatment. For the above-mentioned reasons, after GnRH treatment, it is predicted that the amounts of LH and FSH that reach the luteal cells in the CL with high vascularity are higher than in the CL with low vascularity; consequently, the luteal BFA increase of the pregnant cows occurs.

The LF area was lower in the GnRH group than the control group on Days 7 and 14 (Fig. 2F). In addition, the incidence of an accessory CL on Day 14 was 96.5% in the GnRH group. This result agrees with that obtained in a previous study where the induction rate of an accessory CL was 93% [45]. After administration of the GnRH agonist, the LH concentration increased acutely (LH surge), which was followed by ovulation 26–34 h later [46]. GnRH treatment on Day 5 or Day 6 caused ovulation of the first-wave DF with a diameter ≥ 10 mm in response to LH, with the formation of an accessory CL [37, 45, 47, 48]. Thus, the decrease in the LF area may have been caused by ovulation of the first-wave DF due to an LH surge after GnRH treatment.

In the current study, we observed no increases in the plasma P_4 concentrations and pregnancy rates due to the GnRH treatment (Table 2, Fig. 2C). The results of GnRH effects on the P_4 concentrations and pregnancy rates using cows that were inseminated or received ET are inconsistent [37, 45, 49–53]. Some studies have clearly shown that the plasma P_4 concentrations increased between Days 11 and 16 (Day 0 = estrus) in Holstein heifers and cows administered GnRH on Day 5 [37, 45, 49]. In contrast, Sterry *et al*. [50] showed that GnRH treatment on Day 5 (Day 0 = AI) did not affect the plasma P_4 concentrations from Day 5 to Day 19. In addition, Nishigai *et al*. [51] also failed to observe an effect on the blood P_4 concentration when the recipient cows were treated with GnRH on Day 6 (Day 0 = estrus), and suggested that the blood P_4 concentration was not affected by the accessory CL because the original CL secreted abundant P_4. On the other hand, some reports indicated a positive effect on pregnancy rate with elevating plasma P_4 concentrations [45, 52]. In contrast, Ellington *et al*. [53] reported no significant improvement in the pregnancy rate of GnRH treatment compared with untreated controls. Further investigations of the relationship between GnRH and its reactivity with plasma P_4 for pregnancy are needed in the future.

In conclusion, we demonstrated that GnRH treatment on Day 5 increased the luteal BFA on Days 7 and 14, and improved the accuracy of pregnancy prediction in recipient cows on Day 14. Portable color Doppler machines have recently increased in popularity. Therefore, evaluating the luteal blood flow after GnRH agonist treatment may be useful for early pregnancy diagnosis after ET under field conditions. However, the BFA values may vary depending on the sensitivity and settings of the ultrasound machine. Therefore, the BFA values reported in the current study might not be comparable to those obtained using other ultrasound machines. Further studies are required to investigate the correlations between luteal blood flow, angiogenic factors such as VEGFA and FGF2, and gonadotropins (FSH and LH) after GnRH agonist treatment.

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