

—Original Article—

Ovulation of the preovulatory follicle originating from the first-wave dominant follicle leads to formation of an active corpus luteum

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Abstract. The objective of our study was to compare the characteristics of the corpus luteum (CL) formed after ovulation of the dominant follicle (DF) of the first follicular wave (W1) and those of the CL formed after ovulation of the DF of the second (induced) follicular wave (W2). Non-lactating Holstein cows were used for this study. In Experiment 1, cows were treated with PGF2 α and GnRH on days 6 and 8 (day 0 = day of follicular wave emergence) for W1 (n = 6) and W2 (n = 6), respectively. Dominant follicles were aspirated on day 9 to quantify the amounts of mRNA (*VEGF120*, *VEGF164*, *FGF-2*, *StAR*, *P450-scc* and *3 β -HSD*) in granulosa cells (GC). In Experiment 2, the size and blood flow area of the CL formed after ovulation of the DF in W1 (W1CL; n = 6) and W2 (W2CL; n = 6) (the day of DF ovulation in W1 and W2 was day 10) were evaluated on days 12, 15, 18 and 21. The plasma P4 concentration was measured on days 10 to 21. The amounts of *VEGF164*, *P450-scc* and *3 β -HSD* mRNA were higher (P < 0.05) in the DF in W1, and those of *VEGF120*, *FGF-2* and *StAR* mRNA tended to be higher (P < 0.1) in the DF in W1. The size of the CL was greater in the W1CL on days 15, 18 and 21. The blood flow area of the CL was greater in the W1CL on days 12 and 15. The plasma P4 concentrations were higher in the W1CL. These results indicate that the CL formed after ovulation of the DF in W1 was greater in terms of size, blood flow and plasma P4 concentration.

Key words: Blood flow, Corpus luteum, Cow, Follicular wave, Gene expression

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The corpus luteum (CL) is formed after ovulation of the dominant follicle (DF) following the LH surge at the end of estrus and produces progesterone (P4) for establishment and maintenance of pregnancy. There is evidence indicating that characteristics of the CL, such as size and steroidogenic capacity, are greatly affected by the characteristics of the preovulatory follicle, such as its size and function [1–3]. In our previous study, the size of the DF and blood flow in the wall of the preovulatory follicle was higher in the first follicular wave than in the second [4]. In addition, the concentration of estradiol (E2) in follicular fluid and the amount of *LHr* mRNA in granulosa cells before the LH surge were higher in the first-wave DF than in the second-wave DF [4]. These results suggest that the first-wave DF is more active than the second-wave DF.

Angiogenesis is necessary for formation of the structure of the developing CL and for acquisition of the steroidogenic capacity to secrete P4 [5]. During development of the CL, angiogenic factors, such as vascular endothelial factor (VEGF) and fibroblast growth factor-2 (FGF-2), are expressed in luteinized cells, and these factors promote construction of the CL's capillary network [6]. In addition, the CL has one of the greatest rates of blood flow per unit of tissue

[7], and the blood flow area in the CL is positively correlated with the plasma P4 concentration [8]. Steroidogenic enzymes involved in producing P4 include steroid acute regulatory protein (StAR), P450 side-chain cleavage (P450-scc) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD), and these enzymes are upregulated during the postovulatory period [9].

Our previous finding of a greater amount of *LHr* mRNA expression in the first-wave DF [4] suggests that the higher responsiveness to the LH surge in the first-wave DF may lead to a greater expression of angiogenic factors or steroidogenic enzymes in granulosa cells (GC). It was hypothesized that the CL formed after ovulation of the first-wave DF has a greater size and greater steroidogenic capacity.

However, the effects of preovulatory follicle characteristics on subsequent CL formation were not fully investigated. Therefore, investigation of the functional differences between the CL formed after ovulation of the first-wave DF and second-wave DF may clarify the relationship between the characteristics of the preovulatory follicle and CL function, and this knowledge may reveal the factors that induce a CL with higher fertility.

The objectives of this study were 1) to compare the mRNA expressions of angiogenic factors (*VEGF120*, *VEGF164* and *FGF-2*) and steroidogenic enzymes (*StAR*, *P450-scc* and *3 β -HSD*) in CGs at more than 24 h after the LH surge between the first- and second-wave DF and 2) to compare the characteristics of the CL, such as cross-sectional area, blood flow area and plasma P4 concentration, after ovulation of the DF between the first and second waves.

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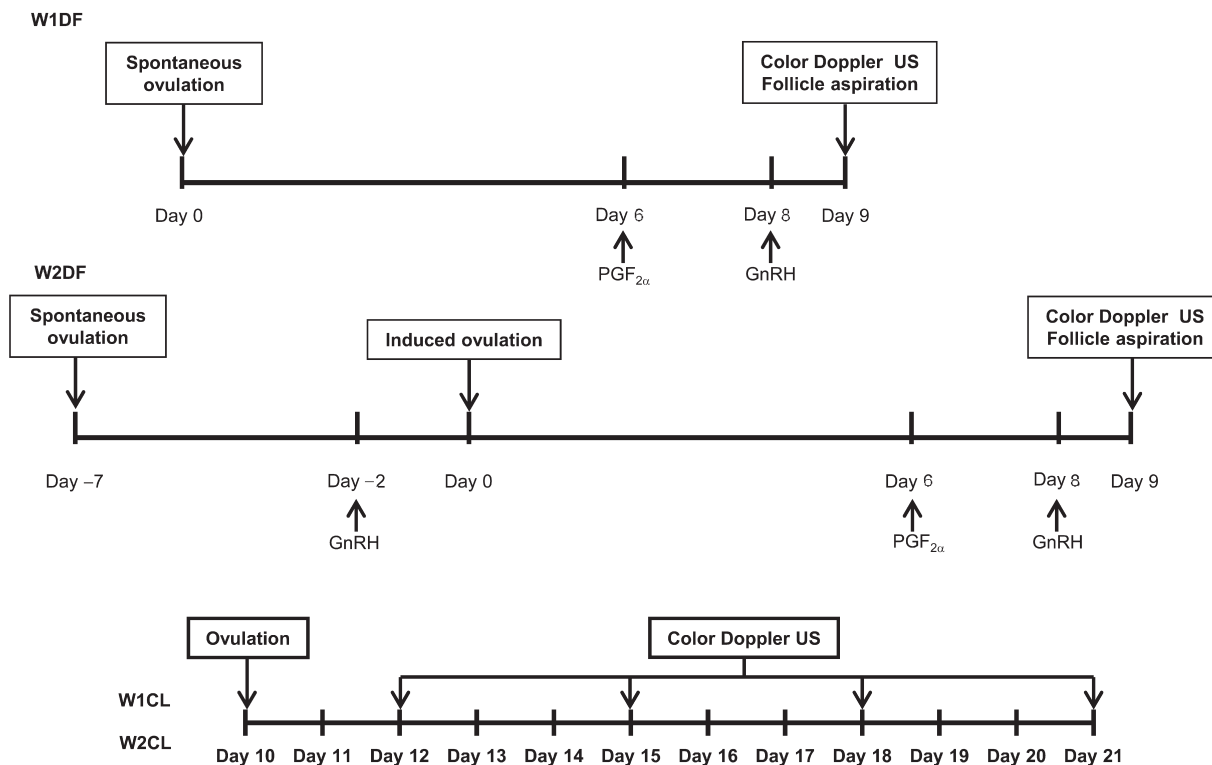


Fig. 1. Schematic diagram of the experimental model for the W1DF, W2DF, W1CL and W2CL. In W1, the day of spontaneous ovulation was defined as day 0. To induce follicular maturation, PGF_{2α} and GnRH were administered on days 6 and 8, respectively. In W2, the day of spontaneous ovulation was defined as day -7. Ovulation from the first-wave DF was induced by GnRH treatment on day -2 to induce a new follicular wave, and induced ovulation was confirmed 36 h after GnRH treatment on day 0. PGF_{2α} and GnRH were administered on days 6 and 8, respectively. In Experiment 1, the dominant follicles (DFs) in W1 and W2 were aspirated on day 9 for collection of follicular fluid and granulosa cells. In Experiment 2, the mean diameter and blood flow in the follicular wall of the DFs in W1 and W2 were evaluated on day 9, and then ovulation was confirmed on day 10 after 36 h of GnRH treatment. Diameter, cross-sectional area and blood flow area of the CL was evaluated on days 12, 15, 18 and 21. Blood samples for hormone assays were collected daily from day 10 to day 21 via the tail vein. Color Doppler US = transrectal color Doppler ultrasonography.

Materials and Methods

Experiment 1

Animals: Six non-lactating Holstein cows (age, mean \pm SEM, 4.0 \pm 1.1 years; range 3–6 years) were used. The cows were kept under the normal management program of the Field Center of Animal Science and Agriculture of Obihiro University and were fed with corn silage, hay and concentrate daily, with free access to water. The cows underwent regular estrous cycles, and were clinically healthy. The experimental procedures complied with the Guide for Care and Use of Agricultural Animals of Obihiro University.

Study design: To compare DF characteristics between the first follicular wave (W1) and second follicular wave (W2), follicular fluid (FF) and GCs were collected after the preovulatory LH surge. In the W1 group, the DF from the first follicular wave after spontaneous ovulation was examined. In the W2 group, the DF from the second (induced) follicular wave was examined. The DFs examined in W1 and W2 were defined as the W1DF and W2DF, respectively. All six cows were included in each group in a crossover design. Therefore, the W1DF and W2DF were examined in different estrous cycles. Figure 1 shows the timetable for the treatment of each experimental

group. Spontaneous ovulation was confirmed in both groups using a transrectal ultrasonic device equipped with a 5.0-MHz linear transducer (HS-101V, Honda Electronics, Toyohashi, Japan) at intervals of 12 h. In the W1 group, the day of spontaneous ovulation was defined as the day of follicular wave emergence (day 0). To induce follicular maturation, prostaglandin (PG)F_{2α} (25 mg dinoprost tromethamine, i.m., per cow; Pronalgon F Vet; Pfizer, Tokyo, Japan) and GnRH (100 μ g fertirelin acetate, i.m., per cow; Conceral; Intervet, Osaka, Japan) were administered on days 6 and 8, respectively.

In the W2 group, the day of spontaneous ovulation was defined as day -7. Ovulation of the first-wave DF was induced by GnRH treatment on day -2 to stimulate the development of a new follicular wave. Induced ovulation, which was confirmed 36 h after GnRH treatment, was defined as the day of follicular wave emergence (day 0). To induce follicular maturation, PGF_{2α} and GnRH were administered on days 6 and 8, respectively.

To collect FF and GCs after the LH surge, W1DFs ($n = 6$) or W2DFs ($n = 6$) were aspirated on day 9 (24 h after GnRH treatment).

Ovarian ultrasonography: The mean diameter of the DF on day 9 was determined using a transrectal ultrasonic device equipped with a 5.0-MHz linear transducer (HS-101V, Honda Electronics,

Table 1. Primers used in real-time PCR

Gene		Sequence of nucleotide (5'–3')	Size (bp)	Annealing temperature (C)	Accession No.
<i>VEGF120</i>	F	CCCAGATGAGATTGAGTTCATTTT	377	56	M32976
	R	GCCTCGGCTTGTCACATTTT			
<i>VEGF164</i>	F	CCCAGATGAGATTGAGTTCATTTT	245	58	M32976
	R	AGCAAGGCCACAGGGATTT			
<i>FGF-2</i>	F	GAACGGGGGCTTCTTCTCT	289	58.2	M13440
	R	CCCAGTTCGTTTCAGTGCC			
<i>StAR</i>	F	GTGCATTTTGCCAATCACCT	203	54.3	M174189
	R	TTATTGAAAACGTGCCACCA			
<i>P450-scc</i>	F	CTGCAAATGGTCCCCTTCT	209	56.3	K02130
	R	CACCTGGTTGGGTCAAACCTT			
<i>3β-HSD</i>	F	TCCACACCAGCACCATAGAA	118	56.3	X17614
	R	AAGGTGCCACCATTTTTCAG			
<i>GAPDH</i>	F	CTCTCAAGGGCATTCTAGGC	160	60	NM001034034
	R	TGACAAAGTGGTCTGTTGAGG			

F, Forward; R, Reverse.

Toyohashi, Japan).

Follicular aspiration and collection of GCs: The DF of each cow was aspirated by transvaginal ultrasound-guided follicle aspiration. An ultrasonic scanner (HS-1500, Honda Electronics) was used for ultrasonic guidance of the aspiration needle and was equipped with a 7.5-MHz transvaginal convex transducer (HCV-3710MV, Honda Electronics) with an attached stainless steel needle guide. All procedures of follicle aspiration were described previously [10]. Follicular fluid was collected into a 1.5-ml plastic tube and brought to the laboratory in ice water. Oocytes were excluded and GCs were collected under a stereomicroscope and placed in a 1.5-ml plastic tube. They were lysed in TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) and stored at -80 C until mRNA isolation. After the removal of GCs, FF was centrifuged at $500 \times g$ for 10 min at 4 C to remove follicular debris and stored at -30 C until the time of hormone assays.

RNA isolation and real-time PCR: Total mRNA was extracted from GCs following the protocol of Chomczynski and Sacchi [11] and treated with DNase using a commercial kit (R01 DNase; Promega, Madison, WI, USA). Single-stranded cDNA was reverse transcribed from total RNA using a first strand cDNA synthesis kit for RT (SuperScript VILO Reaction Mix; Invitrogen, Carlsbad, CA, USA). The RT conditions consisted of 10 min of annealing at 25 C, 60 min of cDNA synthesis at 42 C and 5 min of inactivation at 85 C. Amounts of mRNA of *VEGF120*, *VEGF164*, *FGF-2*, *StAR*, *P450-scc*, *3 β -HSD*, and *GAPDH* were quantified by real-time PCR using a Rotor-Gene Q (QIAGEN, Tokyo, Japan) and commercial kit (Tli RNaseH Plus; QIAGEN). The primers were designed using the Primer3 software based on bovine sequences for real-time PCR (Table 1). The amplification program consisted of 40 cycles of PCR (94 C for 30 sec, annealing temperature for 30 sec, and 72 C for 30 sec). Amounts of mRNA were normalized to *GAPDH*, which was used as an internal control.

Experiment 2

Animals: Six non-lactating Holstein cows (age, mean \pm SEM, 4.0 \pm 1.1 years; range 3–6 years) were used. The cows were kept under the normal management program of the Field Center of Animal Science and Agriculture of Obihiro University and were fed corn silage, hay and concentrate daily, with free access to water. The cows showed regular estrous cycles, and were clinically healthy. The experimental procedures complied with the Guide for Care and Use of Agricultural Animals of Obihiro University.

Study design: The study design was the same as in Experiment 1. However, in this experiment, DFs were not aspirated on day 9, and ovulation of the W1DF and W2DF was confirmed on day 10 after 36 h of GnRH treatment. The mean diameter and blood flow in the follicular wall on day 9 in the W1DF and W2DF were measured by transrectal color Doppler ultrasonography. The cross-sectional area and blood flow of the CL formed after ovulation of the W1DF (W1CL) and W2DF (W2CL) were measured by transrectal color Doppler ultrasonography on days 12, 15, 18 and 21. Blood samples were collected from days 10 to 21 to measure the plasma concentration of P4.

Ovarian ultrasonography: The mean diameter and blood flow in the follicular wall of the DF on day 9 and the cross-sectional area of the CL and blood flow in the CL were assessed using a color Doppler ultrasound instrument (LOGIQ Book XP; GE Yokogawa Medical Systems, Tokyo, Japan) with a 7.5-MHz linear-array transducer (GE Yokogawa Medical Systems). The settings for the color Doppler system were fixed and were the same for all examinations. The maximum cross-sectional area of the DF or CL, including the blood flow infusion point, were evaluated in three different artifact-free images using the largest possible number of color pixels and stored for subsequent analysis.

Evaluation of the cross-sectional area of the CL: The cross-sectional area of the CL was evaluated as follows: cross-sectional area of CL = $\pi/4 \times (\text{mean diameter of CL})^2$. If the CL had a cavity, the cross

sectional area of the CL was evaluated as follows: cross-sectional area of CL = $\pi/4 \times (\text{mean diameter of CL})^2 - \pi/4 \times (\text{mean diameter of cavity})^2$.

Evaluation of blood flow: Stored images were exported to a personal computer and then viewed on a monitor.

Blood flow in the follicular wall was evaluated as follows. Total follicle circumference (FC) and the length of the FC with blood flow signals were measured using Photoshop 5.5 software (Adobe Systems, San Jose, CA, USA). Subsequently, the length of the FC with blood flow signals was divided by the FC. Mean values of three images were used to evaluate blood flow in the follicular wall.

Blood flow area in the CL was evaluated as below. The colored area was selected and changed to a black-and-white image using Adobe Photoshop 5.5 software, and the area was quantified using the PopImaging software (Digital Being Kids, Kanagawa, Japan). Mean values of three images were used to evaluate blood flow in the CL.

Blood sampling: Blood samples were collected into heparinized tubes on all experimental days (days 10–21). Plasma was separated by centrifugation at $2000 \times g$ for 20 min at 4 C and frozen at -30 C until the determination of P4 concentrations.

Hormone assays: Concentrations of P4 and E2 in plasma or FF were determined in duplicates using a second-antibody enzyme immunoassay (EIA). All EIA procedures were as previously described [12]. Steroid hormone assays were performed after extraction with diethyl ether. The recovery rates of P4 and E2 were 87% and 83%, respectively. The standard curve ranged from 0.05 to 50 ng/ml for P4 and, from 2 to 2000 pg/ml for E2. The ED_{50} of the assay was 2.4 ng/ml for P4 and 105 pg/ml for E2. The intra- and inter-assay CVs were 6.4% and 8.6% at 1.5 ng/ml for P4 and 8.1% and 10.6% at 95 pg/ml for E2, respectively.

Statistical analysis

Statistical analyses were performed using StatView Version 5.0 (SAS Institute, Cary, NC, USA). In Experiment 1, amounts of mRNA were compared between groups (W1DF vs. W2DF) using an unpaired Student's *t*-test. In Experiment 2, quantitative end points for mean diameter and blood flow in the follicular wall on day 9 were compared between groups (W1DF vs. W2DF) by an unpaired Student's *t*-test. For assessment of sequential data on cross-sectional area of the CL, blood flow area in the CL and plasma concentration of P4, two-way ANOVA with repeated measures was used to determine the main effects of group (W1CL vs. W2CL) and day (days 10–21) and their interaction. When a significant interaction was detected, Scheffe's *F*-test was used for multiple comparisons test to detect significant differences among groups within days and among days within groups. All data were confirmed as being normally distributed and were analyzed by the *F*-test or Bartlett's test to verify homogeneity of variances. A *P* value of < 0.05 indicated a significant difference, a *P* value of < 0.1 indicated a tendency. Data are presented as the mean \pm SEM.

Results

Experiment 1

Mean diameter of the DF on day 9: The mean diameter of the DF on day 9 tended to be greater ($P < 0.1$) in the W1DF (18.2 ± 0.9

mm) than in the W2DF (16.6 ± 0.5 mm).

Amounts of mRNA in GCs: The amounts of *VEGF120*, *VEGF164*, *FGF-2*, *StAR*, *P450-scc* and *3 β -HSD* mRNA in GCs are shown in Fig. 2. *VEGF120* tended to be greater in the W1DF than the W2DF ($P < 0.1$). *VEGF164* was greater in the W1DF than the W2DF ($P < 0.05$). *FGF-2* tended to be greater in the W1DF than the W2DF ($P < 0.1$). *StAR* tended to be greater in the W1DF than the W2DF ($P < 0.1$). *P450-scc* and *3 β -HSD* were greater in the W1DF than the W2DF ($P < 0.05$).

Follicular fluid concentrations of P4 and E2: The follicular fluid concentrations of P4 and E2 are shown in Table 2. There were no differences between the W1DF and W2DF.

Experiment 2

Mean diameter and blood flow in the follicular wall of the DF on day 9: The mean diameter and blood flow in the follicular wall of the DF are shown in Table 3. Both indexes were greater in the W1DF than in the W2DF ($P < 0.05$).

Cross-sectional area of CL: The cross-sectional area of the CL is shown in Fig. 3. Both main effects (group and day) and group \times day interaction were significant for the cross-sectional area of the CL ($P < 0.01$). The cross-sectional area was greater in the W1CL than the W2CL on days 15, 18 and 21 ($P < 0.05$).

Blood flow area in the CL: The blood flow area in the CL is shown in Fig. 4. Both main effects (group and day) and the group \times day interaction were significant ($P < 0.01$). The blood flow area was higher in the W1CL than in the W2CL on days 12 and 15 ($P < 0.05$).

Plasma concentration of P4: The plasma concentrations of P4 are shown in Fig. 5. Both main effects (group and day) were significant, but the group \times day interaction was not significant. The plasma concentration of P4 increased from day 10 to day 21, regardless of the group ($P < 0.001$). The plasma concentration of P4 was greater in the W1CL than in the W2CL from day 10 to day 21 ($P < 0.001$).

Discussion

In the present study, the amounts of *VEGF120*, *VEGF164*, *FGF-2*, *StAR*, *P450-scc* and *3 β -HSD* mRNA in the GCs of the W1DF were greater than those in the W2DF, and the W1CL had a greater growth rate, blood flow, and the plasma P4 concentration than the W2CL. These results suggested that the W1CL exceeded the W2CL in size, blood flow and steroidogenesis and that the characteristics of the W1CL and the W2CL were already different just before ovulation of the preovulatory follicle.

From Experiment 1, comparison of the mRNA expression levels in GCs collected at 24 h after GnRH treatment between the W1DF and W2DF revealed that the amounts of angiogenic and steroidogenic factors were greater in the W1DF than in the W2DF. These results indicated that the luteinization process may be more active in the W1DF after the LH surge. In our previous study [4], comparison of the preovulatory follicle between W1 and W2, which had not been exposed to the LH surge, revealed that the amount of *LHR* mRNA in GCs was greater in W1 than in W2. It was presumed that the W1DF was more responsive to the LH surge, which may be caused by greater *LHR* mRNA expression in GCs of the W1DF, which may in turn induce active luteinization after ovulation in the W1DF.

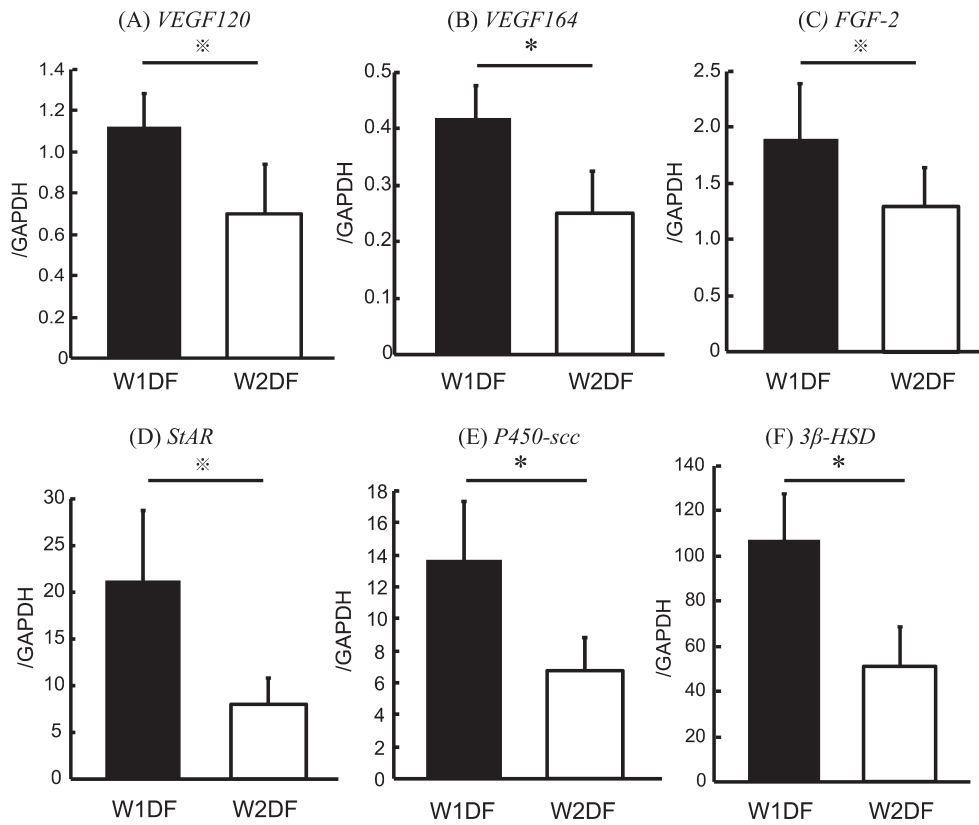


Fig. 2. The relative amounts of mRNA of *VEGF120* (A), *VEGF164* (B), *FGF-2* (C), *StAR* (D), *P450-scc* (E) and *3β-HSD* (F) in the W1DF and W2DF granulosa cells (n = 6/group). In each figure, the black bar indicates the W1DF and the white bar indicates the W2DF. Values are shown as the mean ± SEM. The asterisks denote differences, * P < 0.1; * P < 0.05.

Table 2. Follicular fluid sex steroids concentrations at day 9

	W1DF	W2DF	P-value
P4 (ng/ml)	90.1 ± 11.8	91.4 ± 7.7	NS
E2 (ng/ml)	82.5 ± 20.7	64.1 ± 8.7	NS

Values are shown as the mean ± SEM. The W1DF (n = 6) and W2DF (n = 6) were analyzed. Day 9 was the day after 24 h of GnRH treatment.

Table 3. DF diameter and blood flow area in the follicular wall at day 9

	W1DF	W2DF	P-value
Diameter (mm)	17.7 ± 0.3	15.7 ± 0.5	P < 0.01
Blood flow in the follicular wall (%)	55.3 ± 4.0	36.1 ± 2.3	P < 0.01

Values are shown as the mean ± SEM. The W1DF (n = 6) and W2DF (n = 6) were analyzed. Day 9 was the day after 24 h of GnRH treatment.

The results of Experiment 1 suggested that the size and function of the CL formed after ovulation of the W1DF would be greater than those of the CL formed after ovulation of the W2DF.

In W1, the cross-sectional area of the W1CL was greater during the mid-luteal phase of the CL (days 15, 18, and 21), and the blood

flow area in the CL was greater during the early phase (days 12 and 15); however, there was no difference during the mid-luteal phase, and the plasma concentration of P4 was high throughout the experimental period.

In a previous study, the size of the preovulatory follicle affected the size of the CL formed after ovulation [1]. In the present study, the W1DF was larger than the W2DF, which may have led to the greater size of the W1CL at days 15, 18 and 21. On the other hand, angiogenesis is necessary to form the structure of the developing CL and provide the steroidogenic capacity to secrete P4 [5]. In previous research, local administration of a VEGF antagonist (soluble VEGF receptor) into the preovulatory follicle impaired the subsequent structure and function of the CL [13]. Furthermore, treatment with an FGFR1 inhibitor using bovine luteal cells caused the maximal reduction in total area of endothelial cell networks and reduced the total number of branch points and degree of branching per endothelial cell island [6]. In Experiment 1 of the present work, the expression levels of *VEGF120*, *VEGF164* and *FGF-2* were greater in GCs of the W1DF than in those of W2DF. Greater amounts of angiogenic factor mRNA in GCs of W1 may lead to greater vascularization during the early luteal phase of the W1CL. Active vascularization may cause a greater blood flow area in the W1CL during the early luteal phase (days 12 and 15), and greater CL formation that could

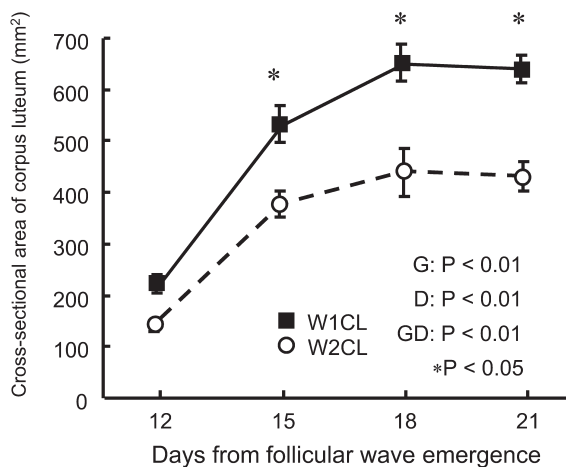


Fig. 3. Comparative changes in the cross-sectional area of CL (mean \pm SEM) of the W1CL (n = 6) and W2CL (n = 6) on days 12, 15, 18 and 21. Day 10 = ovulation day of the DF in W1 and W2. Effect of group (G) ($P < 0.01$), day (D) ($P < 0.01$) and interaction between group and day (GD) ($P < 0.01$). The asterisks denote differences at the indicated time points, * $P < 0.05$.

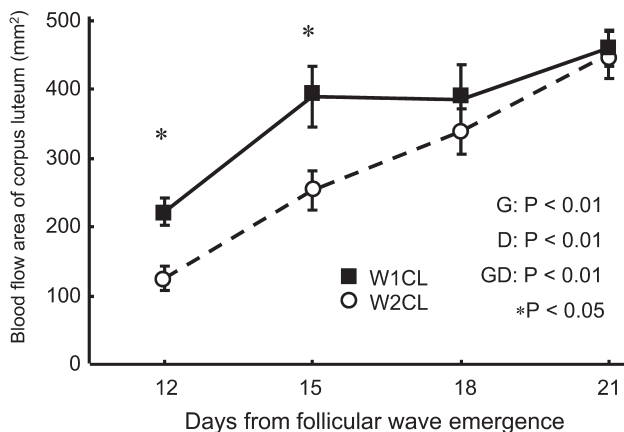


Fig. 4. Comparative changes in blood flow area in the CL (mean \pm SEM) of the W1CL (n = 6) and W2CL (n = 6) on days 12, 15, 18 and 21. Day 10 = ovulation day of the DF in W1 and W2. Effect of group (G) ($P < 0.01$), day (D) ($P < 0.01$) and interaction between group and day (GD) ($P < 0.01$). The asterisks denote differences at the indicated time points, * $P < 0.05$.

lead to a larger W1CL in the mid-luteal phase (days 15, 18, and 21).

Progesterone is synthesized in luteal cells by several steroidogenic enzymes, such as StAR, P450-scc and 3 β -HSD. StAR regulates the transportation of cholesterol from the outer to the inner mitochondrial membrane [9], P450-scc catalyzes the conversion of cholesterol to pregnenolone [9], and 3 β -HSD converts pregnenolone to progesterone [9]. In a previous study, there was a positive relationship between the plasma concentration of P4 and CL size in the mid-luteal phase in cattle [14]. Therefore, it was thought that the greater plasma

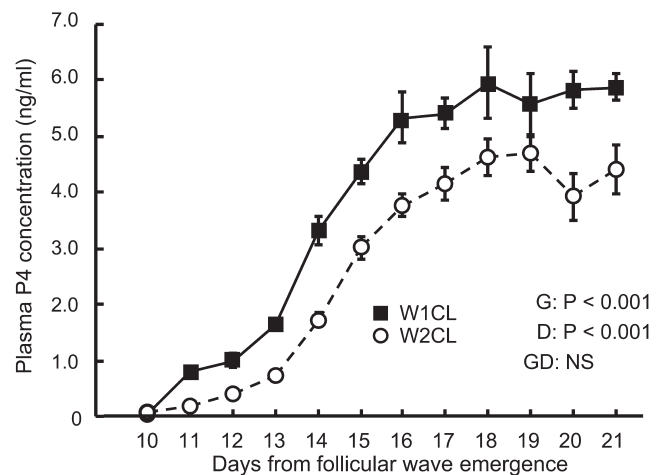


Fig. 5. Comparative changes in the plasma P4 concentration (mean \pm SEM) in the W1CL (n = 6) and W2CL (n = 6). Blood samples were collected daily from days 10 to 21. Day 10 = ovulation day of the DF in W1 and W2. Effect of group (G) ($P < 0.001$) and day (D) ($P < 0.001$).

concentration of P4 in the W1CL may be the result of the greater size of the W1CL during the mid-luteal phase (days 15, 18 and 21). On the other hand, Mann [15] reported that the size of the CL and plasma P4 concentration were not related to each other during the mid-luteal phase. From the results of Experiment 1, the amounts of *StAR*, *P450-scc* and *3 β -HSD* mRNA in GCs were higher in the W1DF than in the W2DF; therefore, it was expected that the steroidogenic capacity of the luteal cells would be greater in the W1CL than in the W2CL. Taking these findings together, the greater size and steroidogenesis in the W1CL may lead to a greater plasma concentration of P4. However, further investigation is required to analyze of mRNA expression levels in luteal cells from luteal biopsy samples to clarify the difference between the W1CL and W2CL.

In previous studies, a lower plasma P4 concentration during follicle development led to an active DF [4, 16]. In the present study, an active CL was formed after ovulation of the DF developed under low plasma P4 concentration conditions. It is well known that the plasma P4 concentration in pregnant cattle is higher than that in nonpregnant cattle [17, 18]. Therefore, it is possible that fertility is higher when artificial insemination is conducted for a preovulatory follicle developed under low plasma P4 concentration conditions. However, the conception rates are lower for insemination of a preovulatory follicle developed under lower plasma P4 concentration conditions [19]. A lower plasma P4 concentration during follicle development disturbs uterus function during subsequent estrous cycle and then induces lower fertility [16]. Therefore, clarification of the appropriate endocrine condition for follicle development and uterus function is needed, and this will make possible to clarify the appropriate endocrine status during follicle development for higher fertility.

In conclusion, compared with W2, the CL formed after ovulation of the DF in W1 was greater in terms of size, blood flow and plasma P4 concentration. From the results of the present study, formation

of the CL was influenced by the size of the preovulatory follicle. In addition, there was a possibility that the size and function of the CL might be affected by the expression levels of mRNA for angiogenic and steroidogenic factors in the GCs of the preovulatory follicle. In addition, the expression levels of these factors may be regulated by the LH responsiveness of the preovulatory follicle.

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