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—Original Article—

Vascular Changes in the Corpus Luteum During Early Pregnancy in the Cow

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Abstract. The present study determined vascular changes in the bovine corpus luteum (CL) at Day 16 (early maternal recognition period) and Day 40 in early pregnancy and compared them to the CL from Day 12 and Day 16 of the estrous cycle. The CLs were analyzed in the central and peripheral regions, where site-depending features of vessels and angiogenic factors are evident. The same protein level of the endothelial cell marker von Willebrand factor was retained in the CL from Day 16 of the estrous cycle to Day 40 of early pregnancy. The protein level of pericytes and smooth muscle cells was determined using smooth muscle α-actin; the level decreased at Day 40 of early pregnancy in both regions of the CL. No significant change in the expressions of vascular endothelial growth factors $VEGF_{164}$ and $VEGF_{120}$ mRNA occurred from Day 16 of the estrous cycle until Day 40 of early pregnancy. Angiopoietin (ANGPT)-2 / ANGPT-1 mRNA ratio (an index of instability of vasculature) increased in the periphery at Day 16 of the estrous cycle and then decreased until Day 40 of early pregnancy. The results suggest that there is no difference in vascular structure between non-pregnant and pregnant luteal tissue during the early maternal recognition period (Day 16). Also, luteal rescue by early pregnancy may be not associated with further blood vessel formation but rather may be related to the decrease of blood vessels per unit of area and blood vessel stabilization in the bovine CL.

Key words: Corpus luteum, Cow, Early pregnancy, Endothelial cells

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The corpus luteum (CL) is temporarily formed in the ovary following ovulation and secretes progesterone (P) to regulate the estrous cycle and to support the establishment of pregnancy. In cows, the CL begins to regress within 17–18 days after ovulation during the estrous cycle but retains a functional lifespan of more than 200 days during pregnancy [1]. Up to 40% of total embryonic loss occurs between Days 7 and 17 of pregnancy [2, 3], which is a period associated with inadequate P concentrations. Thus, some changes of function and morphology in the CL to approve conception are critical during the estrous cycle, the early maternal recognition period and early pregnancy in the cow.

Several distinct cell types, such as small and large luteal cells, vascular endothelial cells and pericytes, are distributed in the bovine CL [4]. More than 50% of cells in the mature CL are of vascular origin [4]. Alterations in luteal vascularity are associated with the luteinization and formation of the CL [5]. Angiogenesis is critical to development of the CL, as an inadequate microvasculature compromises luteal function [6]. The formation of a dense capillary network in the ovary enables the hormone-producing cells to obtain the oxygen, nutrients and also precursors necessary to synthesize and release different hormones essential for maintenance of the ovarian functions. Large luteal blood vessels, i.e., arteriolovenous vessels, have a smooth muscle cell layer and exist in the peripheral, but not central, region of the rabbit CL [7]. Indeed, in the bovine CL, the arteriolovenous vessels exist more in the periphery of the matured CL (mid, late and regressing CL) than in the center region [8]. In the early CL, there are as many arteriolovenous vessels in the periphery as in the center, while more capillaries existed in the center than in the periphery of the mid and late CL [8]. These findings suggest that the central region has the highest density of capillary vessels. Moreover, we have previously shown clear site-dependent features by exerting different effects of prostaglandin (PG) $F_{2\alpha}$ on blood flow and gene expression at the center and periphery of the bovine CL [9].

There is general agreement that a surge of angiogenesis occurs within the CL during its initial development, which is characterized by increased proliferation of endothelial cells and secretion of vascular endothelial growth factors (VEGF), and up-regulation of angiopoietins (ANGPT) [10–13]. There is a discrepancy among species, however, concerning whether or not a second surge of angiogenesis occurs during early pregnancy [10, 11, 13]. Luteal rescue does not appear to be associated with a further burst of angiogenesis in primate [10, 11] and ovine CLs [14].

However, little is known regarding blood vessel stability and its regulation in the bovine CL in the mid to late-luteal phase and early pregnancy during the luteal transition period. In addition, there is no information available about the site-dependent features of vessels and angiogenic factors in the periphery and center region of the CL of early pregnancy in cows. Therefore, the objective of the current study was to obtain the information about vascular changes in

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the bovine CL, distinguishing between peripheral and central parts, at early maternal recognition in the cow (Day 16) and after implantation of the embryo (Day 40), in comparison with the mid and late luteal phases (Day 12 and 16) during the estrous cycle.

Materials and Methods

Animals

Twenty-four normal cyclic, German Holstein cows (age 5.1 ± 0.5 years, body weight 687 ± 23 kg, mean ± SEM) that were non-lactating, barn housed at the Clinic for Cattle, University of Veterinary Medicine Hannover, Germany, and under controlled nutrition were used in the present study. The experimental protocol was approved by the Ethics Committee on Animal Right Protection (Oldenburg, Germany) in accordance with German legislation on animal rights and welfare (file reference number 33.9-42502-04-07/1275).

Experimental design

All 24 cows were selected based on palpation per rectum of genitalia and ovaries to assess normality and the number of follicular waves detected by transrectal ultrasonography (LOGIQe, GE Healthcare, Munich, Germany) with a 5–10 MHz linear probe. In this study, only cows with two follicular waves were selected for investigation of the CL of the estrous cycle to assure the same stage of luteal maturation in each group. Therefore, we checked the condition of the ovary and follicles every two days using ultrasonography during the previous estrous cycle and confirmed the number of follicular waves. Thereafter, the animals were divided into the following four groups: ovariectomy during the estrous cycle (not inseminated) on Days 12 and 16, with five cows on each day, respectively, and ovariectomy of 5 cows after insemination on Day 16. Additionally, a positive control group containing seven animals at Day 40 of pregnancy was included in the study. To determine the exact day of ovulation, all cows received GnRH (0.01 mg Buserelin, 2.5 ml of Receptal™, Intervet, Unterschleißheim, Germany), $PGF_{2\alpha}$ (0.5 mg Cloprostenol, 2.0 ml Estrumate™, Essex Tierarznei, Munich, Germany) seven days later and then GnRH 48 h after $PGF_{2\alpha}$. Only animals that had a preovulatory follicle by the last GnRH application were included in the study. The inseminated animals in the Day 16 and Day 40 pregnant groups were artificially inseminated 12 and 24 hours after GnRH application. Two days after GnRH, ovulation (=Day 1) was confirmed by ultrasonography in all animals.

Blood samples were collected into 10-ml tubes containing 0.3 M EDTA (BD Vacutainer™, Belliver Industrial Estate, Plymouth, UK) at 0800–1000 h daily by puncture of the coccygeal vessels throughout the estrous cycle until the cows were ovariectomized. Day-40 animals were bled once weekly after Day 16. Blood samples were immediately centrifuged for 20 min at 2500 × *g*, and the plasma was then stored at –20 C until assayed.

Collection of ovaries

For logistic and technical reasons, collection of the ovaries was conducted by laparotomy. After administration of epidural anesthesia (4.0 ml 2% procaine hydrochloride, Procasel™, Selectavet, Weyarn-Holzolling, Germany) shaving and aseptic preparation of the lateral region of the right flank, the 20-cm long incision line was infiltrated subcutaneously and intramuscularly with 80 ml of 2% procaine hydrochloride with epinephrine (Isocain™, Selectavet). Additionally, a paravertebral anesthetic block was applied with 100 ml of 2% procaine hydrochloride with epinephrine. After removing the CL-bearing ovary with an effeminator (Reisinger, modified by Richter), the laparotomy was closed in layers.

While pregnancy could only be confirmed by ultrasonography from Day 25 onwards, the Day 16 inseminated cows were slaughtered one day after ovariectomy to collect the uterus for pregnancy detection. At a local abattoir, the reproductive tract was recovered within 20 min after slaughter, and the uterus was ligated cranial of the cervix to prevent embryo loss transported to the laboratory within 30 min of slaughter. This procedure allowed the Day 16 CLs from the inseminated cows to be classified retrospectively. In two animals, no embryo was found; therefore, these CLs were excluded from the experiment.

Processing of the corpus luteum

The CL was enucleated from the ovary and dissected free of connective tissue as in our previous study [15]. Thereafter, the CL was divided lengthwise in halves. Tissue samples were fixed in Bouin's fixative for 24 h at room temperature and then embedded in paraffin wax. Serial sections (5 μm) were mounted onto APS-coated glass microscope slides. The sections were stained with hematoxylin-eosin (H–E) for general histological observations.

In this study, every observation and quantification was performed in the periphery and center of the CL separately as in our previous study [15]. To examine the local effect in the CL, tissue samples were collected from regions designated as the periphery of the CL (in the range of 1 mm from the boundary between the luteal tissue and ovarian parenchyma) and center of the CL (in the range of 1.5 mm from the center section of the CL). The CL tissue samples were collected, minced and immediately placed into a 1.5-ml microcentrifuge tube with and without 0.4 ml TRIzol reagent (Invitrogen, Karlsruhe, Germany) and then stored at –80 C until analysis.

Histology and immunohistochemistry

Light microscopic immunohistochemical staining employing the avidin-biotin peroxidase complex (ABC) method [16] was used as in a previous study [9]. We used polyclonal antibodies for von Willebrand Factor (VWF; Dako, diluted 1:200), which is the marker of endothelial cells, and smooth muscle actin (SMA; Dako, diluted 1:200) which is the marker of smooth muscle cells and pericytes in the present study. As the secondary antibody, we used biotinylated goat anti-rabbit IgG (1:200, BA-1000, Vector Laboratories) for VWF and the negative control and biotinylated goat anti-mouse IgG (1:200, BA-9200, Vector Laboratories) for SMA. Horseradish peroxidase (HRP) conjugated ABC (1:2 dilution, PK-6100, Vectastain Elite® ABC kit, Vector Laboratories) combined with the secondary antibody were applied to tissue slides at room temperature for 30 min. The binding sites were visualized with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris-HCL (pH 7.4) containing 0.02% H_2O_2.

Quantification methods

The positive staining areas for SMA and VWF were extracted using PopImaging (ver. 3.01, Digital Being Kids, Kanagawa, Japan) to calculate the percentage area of the immunostaining (area of the immunostaining divided by the total area measured × 100) as previously described [9]. Areas of positive staining for SMA and VWF were analyzed at × 200 magnification using one section from each animal and five central and peripheral fields per section, respectively.

Western blotting

The CL tissue samples were homogenized in lysis buffer containing 25 mM Tris-HCL (pH 7.4), 0.3 M saccharose, 2 mM Na_2EDTA and Protease Inhibitor Cocktail (Complete, Roche, Mannheim, Germany) and then passed through a 70-μm filter (Cell Strainer, BD Falcon™, Becton, Dickinson and Company, Franklin Lakes, NJ, USA, REF 352350). The proteins were dissolved in sample buffer (0.5 M Tris-HCL (pH 6.8), glycerol, 10% SDS, 0.5% bromophenol blue) and steamed for 5 min. The entire samples were subjected to 5–12% SDS-PAGE for 40–50 min at 200 V. The proteins were transferred to nitrocellulose membranes (Bio-Rad, Tokyo, Japan) for 2–4 h at 30–60 V. The membranes were blocked with 2% nonfat dry milk (Wako, Osaka, Japan) in PBS with 0.5% Tween 20 (Sigma) for 1 h at room temperature. Then, they were incubated with a VWF antibody (1:500 dilution), an SMA antibody (1:1000 dilution), and an anti-β-actin mouse monoclonal clone AC-15 antibody (Sigma, St. Louis, MO, USA. 1:5000 dilution). Thereafter, the membranes were washed three times in PBS with 0.5% Tween 20, HRP-conjugated anti-rabbit or anti-mouse IgG antibodies were added for 1 h at room temperature and the membranes were then washed three times. The signals were detected using an ECL Western Blotting Detection System (GE Healthcare UK, Little Chalfont, UK). The optical density of the immunospecific bands was quantified by means of an NIH Image computer-assisted analysis system.

Progesterone determination

The plasma P concentration was determined by direct enzyme immunoassays (EIA) [17]. The minimum detectable concentration of the assay was 0.3 ng/ml. The intra- and interassay coefficients of variation were 6.2% and 12.5%, respectively. For P determination in the CL tissue samples, homogenized solutions were used as described above for western blotting. Homogenized solutions were diluted 1000 times using assay buffer for steroid EIA (40 mM PBS, 0.1% BSA, pH 7.2), and the 200-μl diluted samples were used for P extraction. The P was extracted using diethyl ether as described previously [17]. The recovery rate of P was 88%. The intra- and interassay coefficients of variation were 6.2 and 9.3%, respectively.

Extraction of RNA

Total RNA was extracted from the CL tissue following the protocol of Chomczynski and Sacchi using TRIzol reagent [18]. The yield of extracted total luteal RNA for each sample was determined by ultraviolet (UV) spectroscopy (optical density, 260). The RNA concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany) at 260 and 280 nm absorbance. The extracted total RNA was stored in RNA storage solution (Ambion, Austin, Texas, USA) at –80 C until use for cDNA production.

Production of cDNA

DNase treatment was carried out using an RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA). Two microliters of the tissue sample were incubated for 30 min at 37 C with 1 μl of RQ1 RNase-Free DNase 10 × Reaction Buffer and 2 μl of 1 μg/μl RNase-Free DNase, respectively. Then 1 μl of RQ1 DNase Stop solution (20 mM EDTA) was added to terminate the reaction, and incubated was performed again for 10 min at 65 C.

First strand cDNA synthesis was conducted according to the commercial protocol described in the SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, California, USA). The first cocktail was prepared using 2 μl for the tissue sample of total RNA, 1.5 μl of 50 ng/μl random primer (Invitrogen), 1.5 μl of 10 mM PCR Nucleotide Mix (dNTP; Roche Diagnostic, Indianapolis, IN, USA) and 12 μl H_2O, making a final volume of 18 μl/tube, and this was then incubated at 65 C for 5 min in a thermal cycler (Bio-Rad, Munich, Germany). The samples were kept on ice, and the second cocktail, 3 μl of 0.1 M DTT (Invitrogen), 1.5 μl of 40 units/μl RNasin® Ribonuclease inhibitor (Promega, Madison, Wisconsin, USA) and 6 μl of × 5 First Strand Buffer (Invitrogen)/tube was added. Then, the samples were again incubated for 2 min at 42 C, and 1.5 μl of 200 units/μl SuperScript™ II Reverse Transcriptase/tube was added. The thermal cycle program was run at 25 C for 10 min, 42 C for 50 min and finally 75 C for 15 min. The synthesized cDNA was stored at –30 C.

Real-time polymerase chain reaction (real-time PCR)

Quantification of mRNA expressions for ANGPT-1, ANGPT-2, Tie-2, $VEGF_{120}$, $VEGF_{164}$ and β-actin was performed using synthesized cDNA via real-time PCR with a LightCycler (Roche Diagnostics, Mannheim, Germany) using a commercial kit (QuantiTect™ SYBR Green PCR, QIAGEN GmbH, Hilden, Germany). The primers were designed using Primer-3 based on the bovine sequences. The amplification program consisted of 15 min activation at 95 C followed by 40 cycles of PCR steps (15 sec denaturation at 94 C, 30 sec annealing at 54–58 C and 20 sec extension at 72 C). For quantification of the target genes, a series of standards was constructed by amplifying a fragment of DNA (400–500 bp) that contains the target sequence for real-time PCR. The primers used for real-time PCR are indicated in Table 1. The PCR products were subjected to electrophoresis, and the target band was cut out and purified using a DNA purification kit (SUPRECTM-01, TaKaRa Bio., Otsu, Japan). Three to five stepwise-diluted DNA standards were included in every PCR run. The values were normalized using β actin as the internal standard. Quantification of mRNA expression was performed using LightCycler Software (Version 3.5; Roche). The housekeeping gene β-actin was used as the internal control to obtain the ratio of each mRNA to β-actin.

Statistical analysis

All data are presented as means ± SEM. For the analysis of the positive areas of VWF and SMA staining, values are presented as a percentage of the total area. The statistical significance of differ-

Table 1. Primers used in real-time PCR

Gene	Sequence of nucleotide[a]	Fragment Size (bp)	Accession no.
$VEGF_{164}$	For 5'-CCCAGATGAGATTGAGTTCATTTT-3' Rev 5'-AGCAAGGCCCACAGGGATTT-3'	245	M32976
$VEGF_{120}$	For 5'-CCCAGATGAGATTGAGTTCATTTT-3' Rev 5'-GCCTCGGCTTGTCACATTTT-3'	377	9M32976
ANGPT-1	For 5'-TGCCAGAACCCAAAAAGGTA -3' Rev 5'-CCCCAACCAATATTCACCAG-3'	155	AF093573
ANGPT-2	For 5'-ACCCTTCAGGTGAACACTGG -3' Rev 5'-CGTGAGGCCTTTAAGGTGAA -3'	179	AF094699
Tie-2	For 5'-GAATGCCCCAAAGGTGATCG -3' Rev 5'-CTTACTTAGAATCTTGGGCG -3'	192	X71424
β-actin	For 5'-CCAAGGCCAACCGTGAGAAGAT-3' Rev 5'-CCACGTTCCGTGAGGATCTTCA-3'	256	K00622

Primer sequences, resulting fragment sizes, and accession numbers of $VEGF_{164}$, $VEGF_{120}$, ANGPT-1, ANGPT-2, Tie-2, and β-actin, which was used as the internal standard. [a]For, forward; Rev, reverse.

ences was assessed by one-way ANOVA followed by Bonferroni's multiple comparison test. Probabilities less than 5% ($P<0.05$) were considered significant.

Results

Progesterone

The plasma P concentrations increased equally in both the cyclic and pregnant animals up to Day 15. In comparison with the pregnant animals, a significant decline in the plasma P concentrations of the cyclic animals occurred on Day 16 (Fig. 1A). On the other hand, the plasma P levels of the pregnant cows remained at the same level until Day 40. No differences could be seen between the four groups in regard to the P content of the luteal tissue (Fig. 1B) on the Day of CL collection.

Endothelial cells, smooth muscle cells and pericytes

The localization of resistant blood vessels and microcapillary vessels in the periphery and center of the CL using immunohistochemistry was investigated to identify blood vessels with endothelial cells (VWF positive) and pericytes and smooth muscle cells (SMA positive). It appears that the intensity of the area positive for endothelial cells decreased from the mid-luteal phase towards early pregnancy in the periphery and center of the CL (Fig. 2A). This decrease of intensity of the positive area for endothelial cells in the periphery and center could be seen on Day 16 of the cycle (Fig. 2B). Thereafter, the same VWF level was retained during early pregnancy on Days 16 and 40. This result is supported by

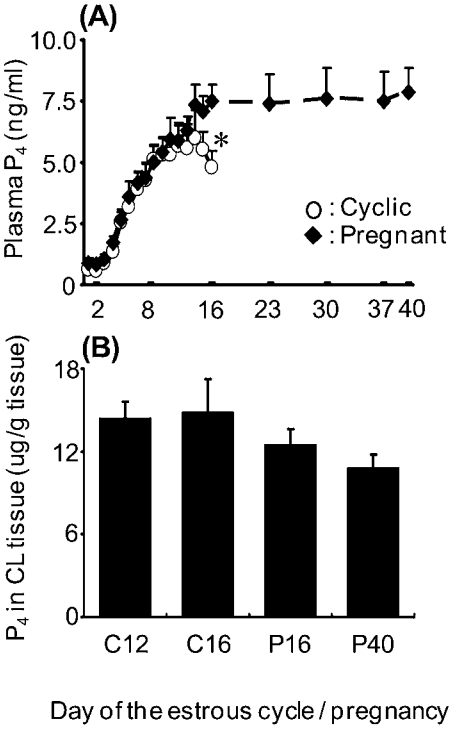

Fig. 1. (A) Changes of plasma P concentrations are indicated with white circles during the estrous cycle and black squares during pregnancy. In the cyclic cow, 10 samples are indicated for cyclic Days 1–12, and 5 samples are indicated for cyclic Days 13–16. In the pregnant cow, 12 samples are indicated for pregnant Days 1–16, and 7 samples are indicated for after pregnant Day 16. (B) Luteal tissue P concentrations during the estrous cycle on Day 12 (C12; n=5) and Day 16 (C16; n=5) and on Day 16 (P16; n=5) and Day 40 (P40; n=7) of pregnancy. All values are shown as means ± SEM. Values with an asterisk (*) are different ($P<0.05$) between the cyclic and pregnant animals at the same time point (A) as determined by ANOVA followed by the Bonferroni multiple comparison test, respectively.

the results of western blotting, which showed that the protein expression of VWF a significant decrease between Day 12 of the cycle and Day 40 of pregnancy. In comparison, no significant difference among Day 16 of the cycle and Days 16 and 40 of pregnancy could be observed (Fig. 2C). Immunostaining for SMA was most evident, predominantly in large luminal vessels but also to a lesser extent in smaller vessels and capillaries, on Day 12 of the cycle (Fig. 3A). The intensity of the positive area and protein expression for smooth muscle cells and pericytes decreased at Day 40 of pregnancy in the periphery as well as in the center of the CL (Fig. 3B and 3C).

Angiogenic factors (VEGF, ANGPT-1/ANGPT-2, and Tie-2)

The mRNA expressions of angiogenic factors in the CL tissue during the transition period from the estrous cycle to pregnancy are shown in Fig. 4. With regard to $VEGF_{164}$ mRNA, there were no apparent differences between the four groups and luteal areas (Fig. 4A). On the other hand, $VEGF_{120}$ mRNA increased in the center at Day 40 of pregnancy compared with Day 12 of the cycle (Fig. 4B). However, no significant change in $VEGF_{120}$ mRNA from Day 16 of the cycle to Day 40 of pregnancy occurred.

In the periphery as well as the center, ANGPT-1 mRNA increased significantly at Day 40 of pregnancy (Fig. 4C), while ANGPT-2 mRNA remained unchanged during the entire transition period (Fig. 4D). The ANGPT-2/ANGPT-1 ratio increased in the periphery at Day 16 of the cycle and then decreased until Day 40 of pregnancy (Fig. 4E). In the central parts, the ANGPT-2/ANGPT-1 ratio decreased only at Day 40 of pregnancy. Tie-2 mRNA decreased at Day 40 of pregnancy (Fig. 4F). In contrast, no change of Tie-2 mRNA was observed in the periphery.

Discussion

This study clarified that the same amount of endothelial cells was retained from the late luteal phase until early pregnancy. On the other hand, a drastic decrease in pericytes and smooth muscle cells occurred in the CLs of early pregnant cows. In addition, the expression of VEGF did not change, and the vasculature had a tendency to stabilize from the late luteal phase of the non-pregnant cycle to early pregnancy. These findings indicate that a new angiogenesis does not occur in the CLs of early pregnant cows.

The reduced immunohistochemical staining and proteins revealed by Western blotting for VWF and SMA toward the late-luteal phase as well as early pregnancy indicate the presence of fewer endothelial cells, pericytes and smooth muscle cells. In regard to luteal endothelial cells, several physical and functional characteristics of luteal endothelial cells of the mid CL are retained through early pregnancy [19]. Lei *et al*. [20] reported that the aver-

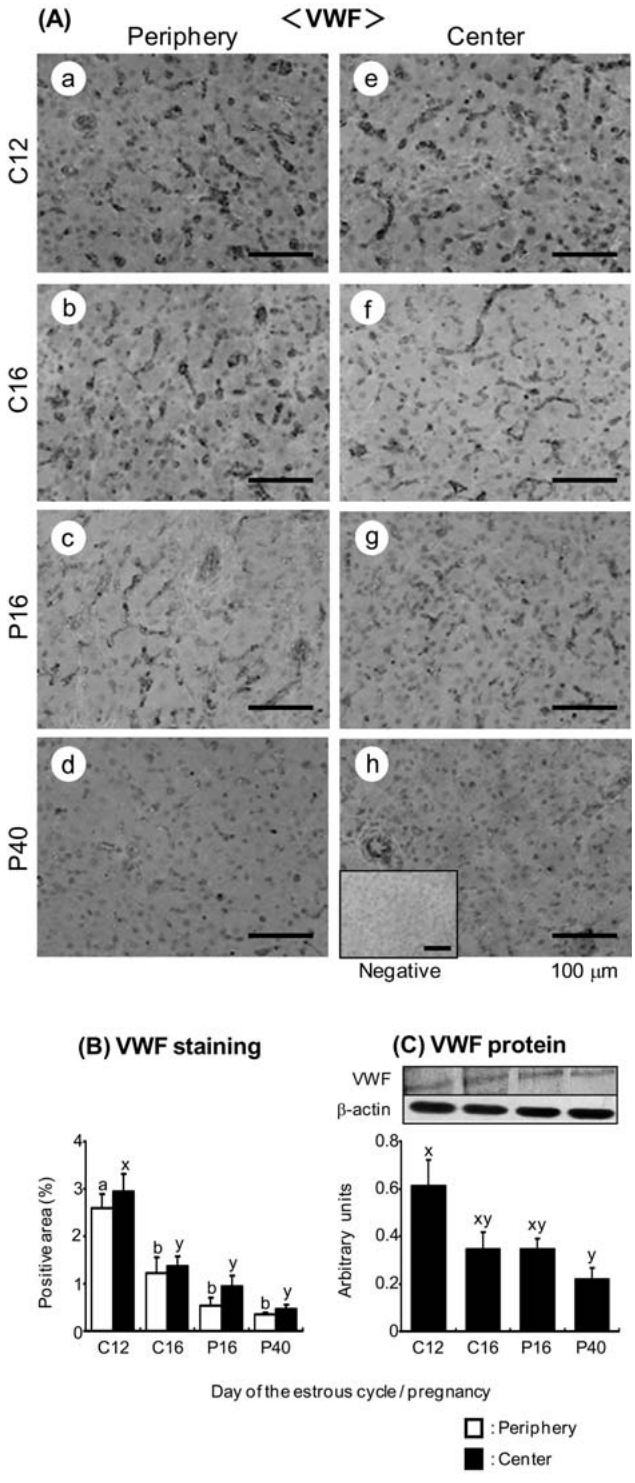

Fig. 2. (A) Immunohistochemical localization of the endothelial cell marker von Willebrand Factor (VWF) in the periphery and center of the CL on Day 12 (C12; a, e) and Day 16 (C16; b, f) of the estrous cycle and Day 16 (P16; c, g) and Day 40 (P40; d, h) of pregnancy. In all cases, the scale bars represent 100 μm, and the original magnification is × 200. (B) The positive staining area of VWF was quantified in the CLs from the different study groups. (C) Western blots for VWF (309 kDa) and β-actin (42 kDa) as the control were loaded for the center of the CL. Relative protein levels of VWF in bovine central luteal tissue from each study group. In the graphs, n=5 for Day 12 and Day 16 of the estrous cycle and for Day 16 of pregnancy, and n=7 for Day 40 of pregnancy. All values are shown as means ± SEM. Different superscript letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by the Bonferroni multiple comparison test.

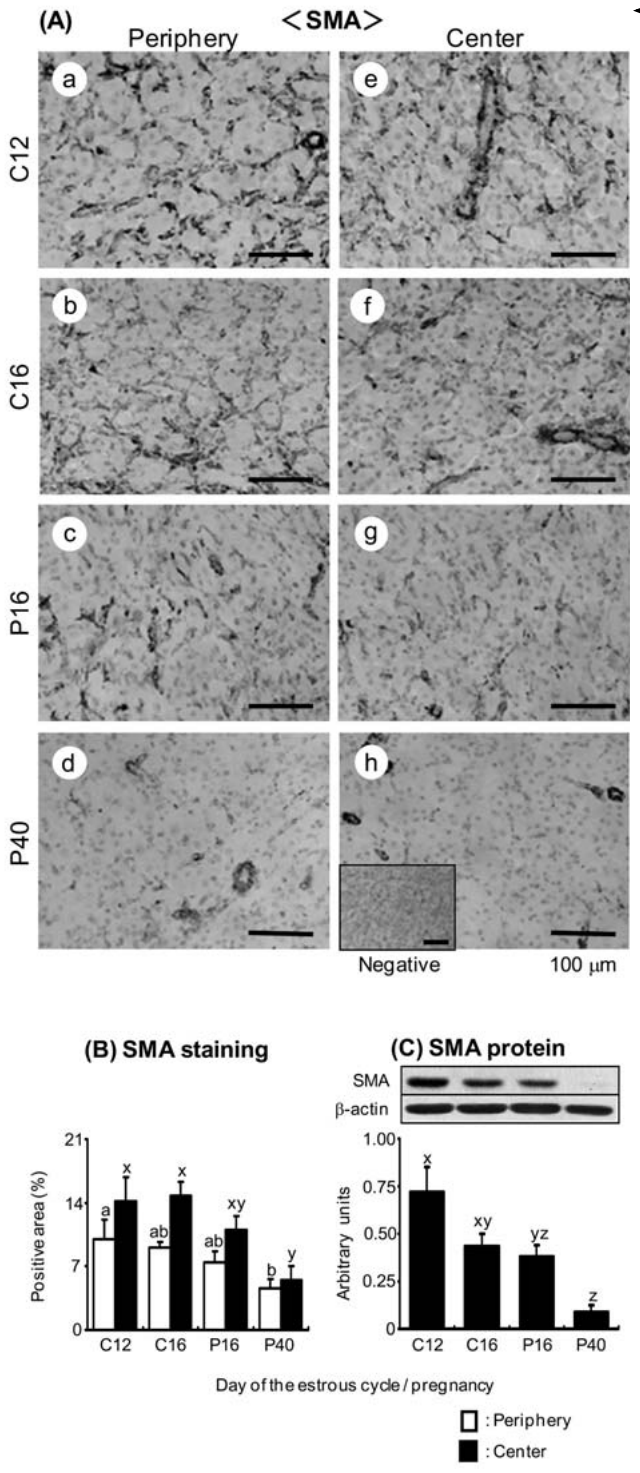

Fig. 3. (A) Immunohistochemical localization of the smooth muscle cell and pericyte marker smooth muscle α-actin (SMA) in the periphery and center of the CL on Day 12 (C12; a, e) and Day 16 (C16; b, f) of the estrous cycle and on Day 16 (P16; c, g) and Day 40 (P40; d, h) of pregnancy. In all cases, the scale bars represent 100 μm, and the original magnification is × 200. (B) The positive staining area of SMA in the CL from the different study groups. (C) Western blots for SMA (42 kDa) and β-actin (42 kDa) as the control were loaded for the center of the CL. Relative protein levels of SMA in bovine central luteal tissue from each study group. In the graphs, n=5 for Day 12 and Day 16 of the estrous cycle and for Day 16 of pregnancy, and n=7 for Day 40 of pregnancy. All values are shown as means ± SEM. Different superscript letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by the Bonferroni multiple comparison test.

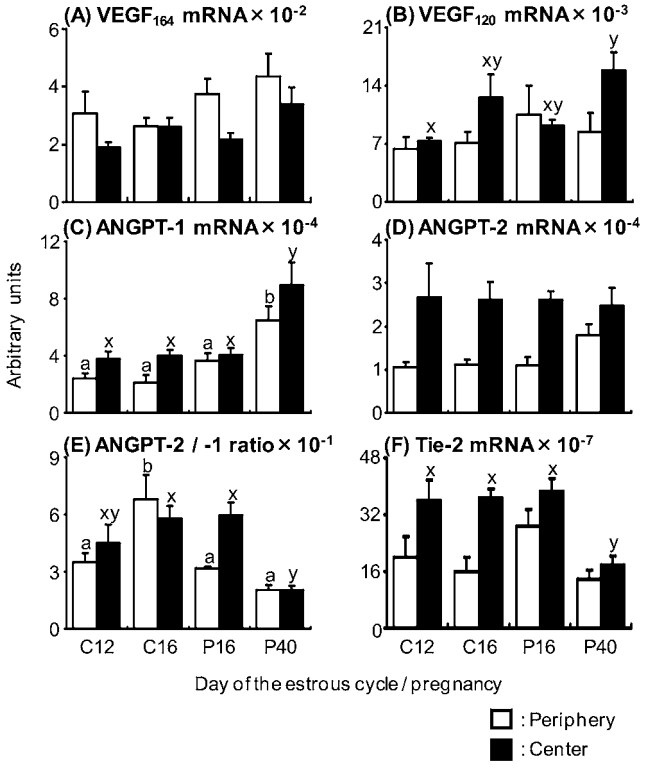

Fig. 4. Relative mRNA levels of $VEGF_{164}$ (A), $VEGF_{120}$ (B), ANGPT-1 (C), ANGPT-2 (D), ANGPT-2/1 ratio (E) and Tie-2 (F) in the bovine CL on Day 12 (C12; n=5) and Day 16 (C16; n=5) of the estrous cycle and on Day 16 (P16; n=5) and Day 40 (P40; n=7) of pregnancy. All values are shown as means ± SEM (relative to the β-actin mRNA levels). Different superscript letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by the Bonferroni multiple comparison test (n=5–7, respectively).

age size of non-luteal cells does not change during the cycle, but it does decrease in the CL of early pregnancy. Because large blood vessels containing smooth muscle cells have a key role in controlling the blood flow to accomplish rapid luteolysis in the cow [9, 21], we speculate that the CL from the early pregnant cow may need a vasculature with less vessels having the ability of autogenic tonus to protect itself from the effect of luteolytic $PGF_{2\alpha}$ on both acute vasodilation and chronic vasoconstriction. Thin-walled cap-

illaries are theoretically unable to control vascular tonus and therefore sustain a "steady-state condition" of blood flow in the CL of early pregnancy.

Recent findings have suggested that ANGPTs and their endothelial cell receptor tyrosine kinase, Tie, may have important roles in the modulation of angiogenesis and angiolysis in the CL [13, 22]. Generally, ANGPT-1 is necessary to maintain and stabilize blood vessels [23]. On the other hand, ANGPT-2, which acts as a natural antagonist for ANGPT-1, appears to cause endothelial cells to undergo active remodeling, thereby destabilizing the vascular structure [23]. Since ANGPT-1 and ANGPT-2 bind to the same receptor, Tie-2, the balance of ANGPT-2 and ANGPT-1 binding to Tie-2 appears to play a crucial role in vascular stability. In the present study, the expression of Tie-2 mRNA did not change during the different stages of the estrous cycle or at Day 16 of pregnancy, but it was significantly decreased on Day 40 of pregnancy. Wulff *et al*. [24] reported that Tie-2 mRNA expression is more intensive in the early CL and rescued CL in the human; however, their data is inconsistent with the data from Sugino *et al*. [12], which showed no significant change in Tie-2 mRNA expression throughout the human menstrual cycle and early pregnancy. Nevertheless, the reduced Tie-2 mRNA expression in our study presumes that the ANGPT-Tie system may be down-regulated at Day 40 of pregnancy in cows when the vascular components are decreasing, suggesting that there is no need for any more vascular changes because the luteal vascular vessels of pregnancy are already stable.

Increasing ANGPT-1 expression induces blood vessel stabilization via recruitment of pericytes during the mid-luteal phase. Endothelial cells in microvessels attract the pericytes to ensheath the capillaries and to influence vessel function [25]. The decreased ANGPT-2/ANGPT-1 ratio at Day 40 of early pregnancy in the present study indicates consistent stability of blood vessels. Moreover, ANGPT-1 plays a role as a survival factor of endothelial cells [26]. Thus, the increase of ANGPT-1 mRNA indicates that ANGPT-1 may support the maintenance of endothelial cells without vessel stabilization through smooth muscle cells or pericytes in consideration of the fact that less SMA was detected on Day 40. Similar to the mitogenic activity in the CL, the ANGPT-2/ANGPT-1 ratio starts to decrease in the periphery of the CL on Day 16 of pregnancy and decreases in both the center and periphery on Day 40. The early decline of the ANGPT-2/ANGPT-1 ratio in the periphery might ensure the stability of large blood vessels to maintain the blood supply just after the onset of the maternal recognition process.

VEGF, the major factor regulating angiogenesis, is a potent mitogen for endothelial cells [27] and a stimulator of vascular permeability [28]. Depending on alternative splicing, there are four different isoforms of VEGF. Mainly the smaller isoforms, $VEGF_{120}$ and $VEGF_{164}$, are found in the bovine CL [29]. In the present study, there was no change in $VEGF_{120}$ and $VEGF_{164}$ mRNA expression between the late luteal phase and early pregnancy. These findings correspond to the non-elevated VEGF expression in the marmoset [30] and bovine CL [29] during early pregnancy. In early human pregnancy, VEGF expression is remarkably high compared with that in the mid-luteal phase [12]. This increase can also be seen in our study for $VEGF_{120}$ on Day 40 of pregnancy compared with Day 12 of the mid-luteal phase. VEGF affects vasodilation and capillary permeability and stimulates endothelial cell growth and angiogenesis in the CL *in vivo* [6]. The possible mechanism underlying the VEGF-induced permeability is based on a rapid formation of fenestrations, which have been observed in the rodent endothelium of small vessels after treatment with human $VEGF_{165}$ [31]. Comparable to other endocrine glands, the capillaries of the CL are fenestrated [32]. The increase of $VEGF_{120}$ on Day 40 of pregnancy could indicate that VEGF may increase vascular permeability and uptake of cholesterol to the luteal cells, resulting in enhancement of luteal function.

As shown in Fig. 1, the plasma P concentrations on Day 16 of the pregnant cows significantly deviated from those of nonbred animals on Day 16. This means that the significant deviation occurred at the time of expected onset of luteolysis. This result correlates with findings of Silva and Costa [33], who indicated that the plasma P concentrations of pregnant heifers (by artificial insemination or embryo transfer) significantly deviate from those of nonpregnant and nonbred heifers on Day 17. On the other hand, no change in the tissue P concentrations could be observed between the CLs of the cyclic and pregnant cows, which is in agreement with a previous report [34]. Although luteal P appears to be produced to maintain early pregnancy during the mid-luteal phase in cyclic animals, there are some independent mechanisms between production of P within the CL and secretion of P into the circulation. However, the mechanism regulating P release into the circulation is still unknown.

In conclusion, the condition of the vascular structure in nonpregnant and pregnant luteal tissue appears to be the same during the early maternal recognition period (Day 16). In addition, luteal rescue is not associated with further blood vessel formation (angiogenesis) but rather relates to the decrease of blood vessels per unit of area in the bovine CL during early pregnancy.

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