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—Full Paper—

Distribution of Arteriolovenous Vessels, Capillaries and eNOS Expression in the Bovine Corpus Luteum During the Estrous Cycle: a Possible Implication of Different Sensitivity by Luteal Phase to $PGF_{2\alpha}$ in the Increase of Luteal Blood Flow

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Abstract. We have shown that luteal blood flow increases in the peripheral vasculature of the mature corpus luteum (CL) prior to the onset of luteolysis in response to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the cow, but this phenomenon does not occur in the early CL. We therefore hypothesize that this acute increase of luteal blood flow occurs by vasodilation of large blood vessels due to local release of nitric oxide (NO). In the present study, we characterized the CL vasculature together with endothelial NO synthase (eNOS) expression during the estrous cycle in the cow. Immunohistochemistry was used to quantify the number of arteriolovenous vessels (surrounded with smooth muscle actin-positive smooth muscle cells), capillaries (with von Willebrand Factor-positive endothelial cells) and eNOS protein. The arteriolovenous vessels existed more in the periphery of the matured CL (mid, late and regressing CL) than in the center region. In the early CL, there were 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. Also, eNOS protein existed in the periphery more than in the center of the matured CL. These results indicate that the early CL has a homogeneous vascular and eNOS distribution. In contrast, the matured CL is a heterogeneous organ having a higher vascular and eNOS distribution in the periphery than in the center. In conclusion, the distribution of arteriolovenous vessels and eNOS in the matured CL was higher in the periphery than in the center of the CL. Thus, this suggests that this structural change from the early (homogeneous) to the mid (heterogeneous) luteal phase is related to the difference in the CL response of blood flow increase due to $PGF_{2\alpha}$, which is only observed in the mature CL.

Key words: Blood vessels, Corpus luteum, eNOS, Luteolysis

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A rich vascular network is established within the corpus luteum (CL) in order to enable it to function as an endocrine gland. Although the CL is constituted of several types of cells, such as luteal cells, endothelial cells and smooth muscle cells, vascular endothelial cells account for up to 50% of the bovine mature CL [1, 2]. Moreover, endothelin-1 (EDN1) and angiotensin II (Ang II) secreted by endothelial cells in the CL have been shown to inhibit progesterone secretion from luteal cells [3–5]. These findings indicate that luteal blood vessels have a key role in regulation of luteal function.

Functionally, luteal blood vessels can be divided roughly into two types of blood vessels. One of these types is an arteriolovenous vessels, i.e., arteriola and venula vessels, which have a smooth muscle layer and receive the vasorelaxant effect. In general, the diameter of an arteriola is about 40 μm (from 20 μm to 100 μm), and the arteriola has an important role in creation of peripheral resistance for local blood flow. In the circulation of blood, the arteriola connects to microcapillary vessels followed by venulae (diameter: about 30 μm) [6]. The venula also has pericyte and/or smooth muscle cells and a vascular tonus function, which includes vasodilation. Indeed, a nitroglycerin, strong vasodilator, can induce vasodilation in not only the arteriola but also in the venula [7]. The other type is capillary vessels, which do not have a smooth muscle layer and receive little vasorelaxant effect. It has been reported that the number of arteriolovenous vessels in the CL increases from the early to mid luteal phase [8, 9]. Additionally, in the rat and rabbit CL, arteriolovenous vessels existed in the periphery of the CL [10, 11], and luteal blood flow is observed mainly in the periphery of the bovine CL in color Doppler images [12, 13]. These findings suggest that the CL is a heterogeneous organ in terms of the distribution of arteriolovenous and capillary vessels.

It has been reported in the ewe that blood flow to the CL decreases dramatically in association with luteolysis [14]. More precisely, our recent study indicated that blood flow in the periphery of the CL drastically and transiently increased at 0.5–2 h after $PGF_{2\alpha}$ injection (the dose of $PGF_{2\alpha}$ analogue recommended by the manufacture) in the mid luteal phase and thereafter gradually decreased over the course of luteolysis [12]. In contrast, $PGF_{2\alpha}$ induces neither luteal blood flow increase nor luteolysis during the

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early luteal phase [12]. Thus, the acute increase of luteal blood flow induced by $PGF_{2\alpha}$ may be one of the earliest physiological signs for the luteolytic cascade.

We recently demonstrated that the increase in luteal blood flow in response to $PGF_{2\alpha}$ is induced by vasodilation due to the local action of luteal nitric oxide (NO) [15]. NO is well known as a potent vasodilator, produced by endothelial NO synthase (eNOS) and inducible NO synthase (iNOS), and the bovine CL expresses both types of NOS [16]. Furthermore, an immunohistochemical study has shown that the bovine CL expresses NOS at the highest level during the late luteal phase of the estrous cycle [16]. Actually, NO inhibits progesterone secretion from luteal cells [17], and an NOS inhibitor prolongs the estrous cycle and delays luteolysis induced by $PGF_{2\alpha}$[16].

We hypothesized that more arteriolovenous vessels, which are capable of vasodilation, should exist in the periphery of the mid CL and that therefore $PGF_{2\alpha}$ could induce an increase in luteal blood flow in the arteriolovenous vessels, where eNOS is dominantly expressed compared with the center of the mid CL; they are thereby more sensitive to $PGF_{2\alpha}$ in vasodilating response. In the present study, we therefore examined the localization of arteriolovenous vessels, capillaries and eNOS expression in the cyclic bovine CL using immunohistochemistry.

Materials and Methods

Immunohistochemistry

Ovaries with CLs were collected from cows at a local abattoir within 10–20 min after slaughter. The ovaries were kept in ice-cold phosphate-buffered saline (PBS, pH 7.2) until preparation at the laboratory. The luteal stage was carefully defined by macroscopic observation of the ovaries (follicles and CL) and described in a previous study [18]. The CLs were divided into four groups as follows: early (Days 2–5; n=5), mid (Days 8–12; n=5), late (Days 15–17; n=5) and regressing (after Day 18; n=5). The CLs were enucleated from the ovary and dissected free of connective tissue. 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.

Light microscopic immunohistochemical staining employing the avidin-biotin peroxidase complex (ABC) method [19] was used as in the previous study [20]. We used polyclonal antibodies for von Willebrand Factor (vWF; Dako, diluted 1:200), which is the marker of endothelial cells, smooth muscle actin (SMA; Dako, diluted 1:200), and eNOS (Affinity BioRegents™, PA1-037, diluted 1:50) in the present study. As the secondary antibody, we used biotinylated goat anti-rabbit IgG (1:200, BA-1000, Vector Laboratories) for vWF, eNOS 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.

Decision of the observation area between the periphery and the center of the CL

In this study, every observation and quantification was performed in the periphery and center of the CL separately as in our previous study [20]. To examine the local effect of $PGF_{2\alpha}$ 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).

Measurement of the cross-sectional area of the large luteal cell (LLC)

The cross-sectional area of an LLC in each luteal phase was measured separately in the periphery and center of the CL. Five sites were randomly chosen from the pictures of the H-E stained sections among each luteal phase and each luteal site (periphery and center). Five LLCs from each chosen area were measured. The cross-sectional area of an LLC was calculated by assuming that the shape of the cell was an ellipse ($S=\pi ab$, a=major axis, b= minor axis).

Quantification of vWF-positive blood vessels

Cells positive for vWF were identified as vascular endothelial cells. A separated block of several endothelial cells was defined as one blood vessel. The number of blood vessels was counted within a unit area (40,000 μm^2) in the microscopic field at × 200 as described in a previous study [15]. Counting was performed on five randomly chosen areas within the periphery of the CL and in the center of the CL. Quantification was performed independently by three observers. The mean of the three observers' data was calculated for each histological section, and the mean of the three observers' related means was used as a single observation.

Quantification of arteriolovenous vessels

In counting of arteriolovenous vessels, arteriolovenous vessels were identified as SMA-positive vascular smooth muscle cells. A vascular lumen that was perfectly covered with a smooth muscle layer was defined as one arteriolovenous vessel. Moreover, the diameter of an arteriolovenous vessel was defined as being from 20 to 50 μm. The number of SMA-positive blood vessels was counted within a unit area (40,000 μm^2) in the microscopic field at × 200 as described in a previous study [15]. The counted blood vessels included SMA-positive pericytes and vascular smooth muscle cells. As for vWF-positive vessels, quantification was performed independently by three observers. The mean of the three observers' data was calculated for each histological section, and the mean of the three observers' related means was used as a single observation.

Calculation of the number of capillaries

The number of capillaries was computed by subtracting the number of SMA-positive blood vessels from the number of vWF-positive blood vessels. The calculation was performed within each of five different fields, and the mean of the five different fields was

used as a single observation.

Percentage area of eNOS immunostaining

The positive staining areas were extracted using PopImaging (Digital Being Kids, Ver. 3.01, Japan) to calculate the percentage area of immunostaining (area of the immunostaining divided by the total area measured × 200) as previously described [15, 21]. Areas were analyzed at 200 × magnification using one section from each animal and five fields per section. The results were expressed as percentage means ± SEM per unit area.

Statistical analysis

All data are presented as means ± SEM. Means of the weight of the CL, number of luteal vessels and percentage of immunohistochemical staining areas during the luteal phase were analyzed by ANOVA followed by Bonferroni's multiple comparison test. Probabilities less than 5% ($P<0.05$) were considered to be significant.

Results

Weight of the CL during the luteal phase

The weight of the CL in the mid (7.9 ± 0.3 g) and late luteal phase (7.7 ± 0.8 g) was about four times heavier than in the early luteal phase (2.1 ± 0.4 g; $P<0.05$, Fig. 1A). The weight of the CL in the regressing luteal phase (3.9 ± 0.3 g) was half of that of the mid and late luteal phase ($P<0.05$, Fig. 1A).

Change of the cross-sectional area of the LLC

In the periphery of the CL, the cross-sectional area of the LLC increased gradually from the early to late luteal phase (Early, 140.0 ± 2.4 μm^2; Mid, 369.7 ± 15.2 μm^2; Late, 576.9 ± 37.1 μm^2; $P<0.05$, Fig. 1B). In the regressing luteal phase in the periphery of the CL, the cross-sectional area of the LLC was half as large as that in the late luteal phase. In the center of the CL, the cross-sectional area of the LLC in the mid luteal phase was about four times larger than that of the early luteal phase (Early, 138.4 ± 17.7 μm^2; Mid, 539.5 ± 39.4 μm^2; $P<0.05$, Fig. 1B). Thereafter, the size of cross-sectional area maintained in the late luteal phase decreased by half in the regressing luteal phase compared with the late luteal phase (Late, 620.2 ± 27.7 μm^2; Regressing, 324.3 ± 43.7 μm^2). Comparing the periphery with the center of the CL, the cross-sectional area of the LLC in the center of the mid CL was larger than that in the periphery of the mid CL ($P<0.05$).

Distribution of the arteriolovenous and capillary vessels during the luteal phase

Figure 2A shows the changes of the arteriolovenous vessels throughout the luteal phase. In the periphery of the CL, the number of arteriolovenous vessels increased during the early to mid luteal phase ($P<0.05$) and thereafter decreased in the late luteal phase ($P<0.05$). During the regressing luteal phase, the number of arteriolovenous vessels increased again ($P<0.05$). In the center of the CL, the number of arteriolovenous vessels was maintained from the early to the mid luteal phase, and thereafter, decreased in the late luteal phase. However, the number of arteriolovenous vessels in the regressing luteal phase was double that of the late luteal phase ($P<0.05$). There were many arteriolovenous vessels distributed in the periphery of the CL compared with the center during the mid to regressing luteal phase. Figure 2C-2H shows the immunohistochemical staining for SMA in the periphery and center of the CL.

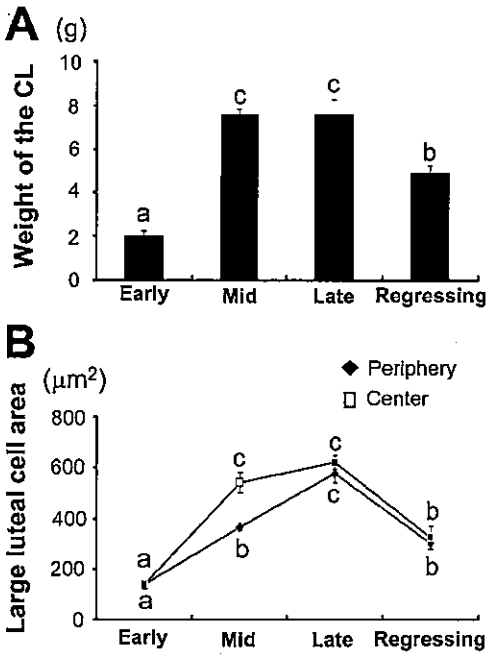

Fig. 1. Changes of the weight of the CL and the cross-sectional area of the large luteal cells throughout the luteal phase. Fig. 1A indicates the changes of the weight of the CL, and Fig. 1B indicates the changes of the cross-sectional area of the large luteal cells throughout the luteal phase. E: Early luteal phase (Days 2–5); M: Mid luteal phase (Days 8–12); L: Late luteal phase (Days 15–17); R: Regressing luteal phase (Days 18–21). In Fig. 1B, black diamonds indicate the periphery of the CL, and white squares indicate the center of the CL. Means ± SEM are presented (n=5). Different superscripts indicate statistically different values ($P<0.05$).

Figure 2B shows the changes of the capillary vessels throughout the luteal phase. In the periphery of the CL, capillaries decreased during the early to mid luteal phase ($P<0.05$). Thereafter, they increased three-fold during late luteal phase ($P<0.05$) and decreased again in the regressing luteal phase ($P<0.05$). In the center of the CL, capillaries increased during the early to mid luteal phase ($P<0.05$). Thereafter, the number of capillaries were maintained until the late luteal phase and rapidly decreased in the regressing luteal phase ($P<0.05$). There were many capillaries distributed in the center of the CL compared with the periphery during the mid to late luteal phase. Figure 2I-N shows the immunohistochemical staining for vWF in the periphery and center of the CL, in which vWF was detected during the early to late luteal phase, although it decreased drastically in the regressing luteal phase. In addition, capillary vessels in the center were detected more during the mid and late luteal phase than in the periphery of the CL.

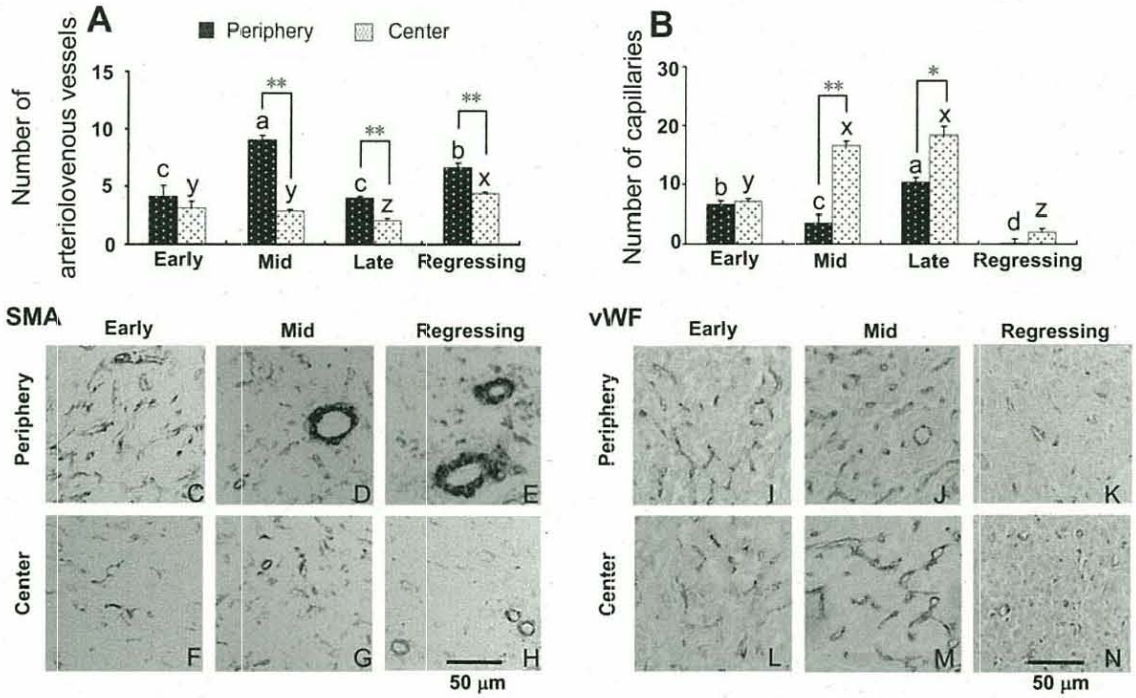

Fig. 2. Changes of arteriolovenous and capillary vessels in the periphery and the center of the CL throughout the luteal phase. Fig. 2A indicates the changes of the number of arteriolovenous vessels, and Fig. 2B indicates the changes of the capillary vessels in the periphery and center of the CL throughout the luteal phase. In each figure, the black bars indicate the periphery of the CL, and the gray bars indicate the center of the CL. Different superscripts indicate statistically different values ($P<0.05$). Fig. 2C-H show the immunohistochemical staining of SMA in the periphery and center of the CL during the early luteal phase (C and F), mid luteal phase (D and G) and regressing luteal phase (E and H). Fig. 2I-N show the immunohistochemical staining of vWF in the periphery and center of the CL during the early luteal phase (I and L), mid luteal phase (J and M) and regressing luteal phase (K and N). Scale bars: 50 μm.

Distribution of eNOS during the luteal phase

Figure 3A shows the changes in the rate of the eNOS-positive stained areas throughout the luteal phase. In the periphery of the CL, expression of eNOS was higher in the early and mid CL and thereafter decreased in the late and regressing luteal phases compared with that in the early and mid luteal phases ($P<0.05$). In the center of the CL, high expression of eNOS was detected in the center of the early luteal phase, thereafter decreased during the mid luteal phase ($P<0.05$) and then was maintained at a stable level until the regressing luteal phase. Although the expression levels of eNOS were the same in the periphery and center of the early CL, there was more eNOS distributed in the periphery of the CL compared with the center during the mid to the regressing luteal phase. Figure 3B–F shows the immunohistochemical staining for eNOS in the periphery and center of the CL.

Discussion

Consistent with the lack of effect of $PGF_{2\alpha}$ on luteal blood flow in the early CL, $PGF_{2\alpha}$ did not increase eNOS expression in the early CL. In contrast, $PGF_{2\alpha}$ acutely stimulated the expression of eNOS in the periphery, but not in the center of the mid CL 30 min after $PGF_{2\alpha}$ administration. Moreover, $PGF_{2\alpha}$ induces an acute increase in NO in the ovarian circulation [22]. We considered that there were several possibilities concerning why $PGF_{2\alpha}$ can induce luteal blood flow increase only in the periphery of the mid CL. 1) In the early CL, the number of arteriolovenous vessels was fewer than in the mid CL, and the vessels were distributed equally in the periphery and center of the CL. On the other hand, in the matured CL, more arteriolovenous vessels were presented in the periphery compared with the center of the CL. 2) In the present spontaneous CLs, eNOS was localized with higher intensity in the periphery than in the center in the matured CLs. However, in the early CLs, the number of arteriolovenous vessels was fewer than in the mid CLs, and the vessels were distributed equally in the periphery and center of the CL. 3) The early CL produces a higher amount of intraluteal $PGF_{2\alpha}$ than the mid CL, suggesting that desensitization of exogenous $PGF_{2\alpha}$ occurs in the early CL [23, 24]. These changes in the distribution of the vasculature and eNOS expression may be one of the reasons why $PGF_{2\alpha}$ becomes capable of inducing one of the signs of early luteolytic events (an increase in luteal blood flow in the mid luteal phase but not in the early luteal phase), probably by stimulating eNOS expression in the arteriolovenous vessels. Therefore, a heterogeneous vascular structure may be directly related to luteolytic ability, and $PGF_{2\alpha}$ may have a site-restricted action depending on not only the luteal phase but also on

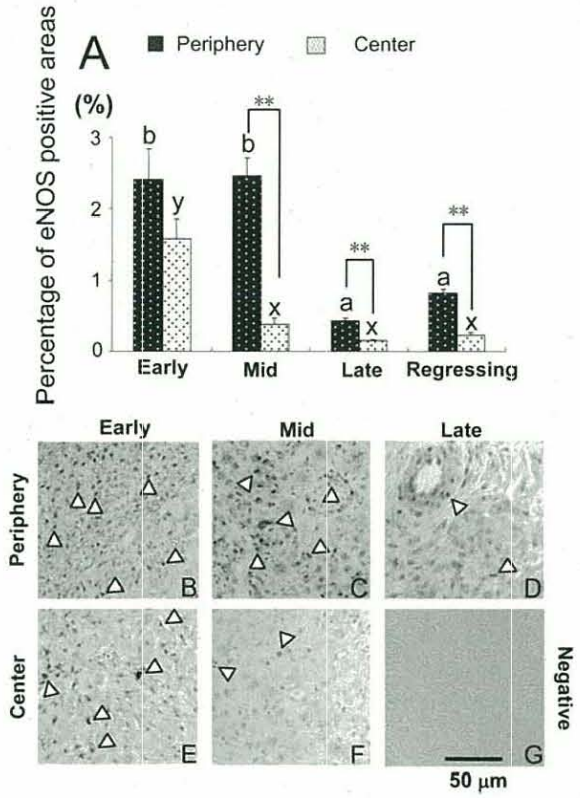

Fig. 3. Changes of the eNOS-positive stained areas and distribution in the periphery and the center of the CL throughout the luteal phase. Fig. 3A indicates the changes of the eNOS-positive stained areas in the periphery and center of the CL. In the figure, the black bars indicate the periphery of the CL, and the gray bars indicate the center of the CL. Different superscripts indicate statistically different values ($P<0.05$). Fig. 3B–F show the immunohistochemical staining of eNOS in the periphery and center of the CL during the early luteal phase (B and E), mid luteal phase (C and F) and late luteal phase (D). Fig. 3G shows the negative control. Scale bars: 50 μm.

the region in the CL.

A rich capillary network is established in the center of the matured CL. In the present study, the number of capillaries increased in the center of the CL but not in the periphery from the early to mid luteal phase. Behind the center of the CL, the number of capillaries in the periphery of the CL increased in the late luteal phase. These differences in timing of the start of increase in cell numbers may be due to heterogeneity in the mitosis of luteal endothelial cells [25]. In fact, from the early to mid luteal phase, the capillary network increases as a result of conspicuous proliferation of endothelial cells [25]. Taken together, the developmental speed of the CL and the timing of angiogenesis within the CL may be regulated differently in the periphery and center regions. The capillary network appears to grow earlier and faster in the center of the CL, followed by the periphery of the CL. In the present study, as synchronized with capillary growth, the cross-sectional area of the large luteal cell also increased faster in the center compared with the periphery of the mid CL. Thereafter, in the late luteal phase, the cross-sectional area of the large luteal cell in the periphery of the CL increased to the same level as that of the center. These findings suggest that the growth of luteal cells may depend on capillary growth because a fully developed capillary network is necessary for supply of nutrition to support the growth of luteal cells. Thus, it is highly possible that the speed of the constructive development of the bovine CL is different between the periphery and center of the CL and is received in part of the different regulation.

The bovine CL has two types of blood vessels, arteriolovenous vessels and capillary vessels. Interestingly, although arteriolovenous vessels increased both in the periphery and center of the CL from the late to regressing luteal phase, the capillary vessels drastically decreased. Indeed, previous reports have indicated that non-capillary vessels having smooth muscle layers increases markedly in the regressing CL compared with the late CL in the cow [8, 26]. During $PGF_{2\alpha}$-induced luteolysis in the sheep, the endothelial component of the vascular bed decreases, whereas high numbers of smooth muscle cells are maintained during luteal regression, suggesting that pericytes and vascular smooth muscle cells have a role in luteolysis [27]. Therefore, capillary vessels seem to regress rapidly in the regressing CL due to shut-off of blood supply by vasoconstriction of arterioles due to factors such as EDN1 and Ang II. On the other hand, large arteriolovenous vessels remained intact and exhibited a thickening wall in the regressing CL, suggesting that arteriolovenous vessels may have a role in the expeditious disrupture of the bovine CL. Further studies will be needed to clarify the physiological meaning for the increase in arteriolovenous blood vessels in the regressing CL.

It is well considered that spontaneous luteolysis induced by endogenous $PGF_{2\alpha}$ and pharmacological luteolysis induced by administration of exogenous $PGF_{2\alpha}$ are different [28–30]. In fact, Skarzynski *et al.* recently reported that estrus synchronization, depending on the method and $PGF_{2\alpha}$ analogue, may induce lower P production and decrease the sensitivity of the CL to luteotropic factors compared with those in the spontaneous estrous cycle in the cow [30]. During $PGF_{2\alpha}$-induced luteolysis in cattle, systemic $PGF_{2\alpha}$ treatment induces a transient increase in luteal blood flow [12, 15]. Additionally, during spontaneous CL regression in the cow, an acute increase in luteal blood flow in the periphery of the mature CL is correlated with pulsatile increases in plasma PGFM (a metabolite of $PGF_{2\alpha}$ produced by the uterus) [15, 31, 32]. A recent report by Ginther *et al.* provided additional evidence showing that CL blood flow increases with each PGFM pulse during spontaneous luteolysis in cattle [32], but not in mares [33]. Although the luteolytic phenomena observed in spontaneous (endogenous) and $PGF_{2\alpha}$-induced (exogenous) luteolysis are not completely the same because excessive $PGF_{2\alpha}$ doses may stimulate nonphysiologic responses [29], these observations indicate that the phenomenon of an increase in luteal blood flow and its mechanisms may be similar in spontaneous and $PGF_{2\alpha}$-induced luteolysis in the cow.

In the present study, a high expression of eNOS was detected in the center of the early CL, although it decreased in the mid, late and regressing luteal phases, confirming previous studies [34, 35]. It has been reported that growth and development of new capillaries occurs in the early CL [36]. In addition, NO has been reported to

promote angiogenesis in human vascular endothelial cells [37]. Also, a previous study has suggested that eNOS has a cooperative effect for angiogenesis with VEGF, a potent angiogenic factor [38]. Thus, luteal NO may act as a luteotropic rather than luteolytic factor in the early CL. On the other hand, it has been reported that an NO donor inhibits progesterone secretion [17] and induces apoptosis [39] of mid luteal cells. These results suggest that luteal NO has dual functions according to the luteal phase.

Shear stress is generated when the blood flow volume and blood pressure are increased [40]. Shear stress is the frictional tangential force imposed on the vessel wall when blood flows through the vessel. Shear stress modulates many physiological processes and is a potent stimulator for NO production and vasodilation [41]. In fact, NO production is elevated by shear stress in a graded fashion in ovine fetoplacental artery endothelial cells [42, 43]. The acute burst of NO production may relate to an increase in eNOS activity, probably via an increase in intracellular Ca^{2+} and phosphorylation of eNOS [43]. It is possible that luteolytic pulses of $PGF_{2\alpha}$ stimulate eNOS activity by inducing intracellular Ca^{2+} and simultaneously increasing luteal blood flow/shear stress in the periphery of the CL. Although we speculated that luteal blood flow and shear stress generated by NO action may have the potential to regulate luteolysis by stimulating other potential luteolytic mediators, such as EDN1 in the cow [15], precise evaluation of the physiological impact of the blood flow increase requires further investigation.

In conclusion, the results indicated that the expression pattern of luteal vasculatures and eNOS was different during the luteal phase in the cow as follows: (1) more arteriolovenous vessels existed in the periphery compared with the center of the mid, late and regressing CL; (2) a larger number of capillaries existed in the center compared with the periphery of the mid and late CL; (3) many eNOS-positive areas were observed in the periphery of the mid, late and regressing CL, where many arteriolovenous vessels were observed; and (4) in the early CL, both the periphery and center of the CL contained similar distributions of capillaries, arteriolovenous vessels and eNOS expression. Thus, this structural change from the early (homogeneous) to mid (heterogeneous) luteal phase is related to the difference in the CL response of blood flow increase due to $PGF_{2\alpha}$, which is only observed in the mature CL.

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