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In Vivo Studies on the Vascular Function in the Bovine Ovary: Determination of Blood Flow and Hormonal Secretion in the Follicle and Corpus Luteum

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A transrectal color Doppler ultrasonography was used to assess changes in the ovarian structures and to determine blood flow that take place in the follicle wall and within the corpus luteum (CL) during specific physiological events such as ovulation, CL development, and CL regression in cows. To investigate the local release of vasoactive peptides, steroid hormones, and prostaglandins (PGs) in each ovarian structure, the capillary membranes (0.2 mm diameter and 5-10 mm length) of a microdialysis system (MDS) were implanted surgically implanted into the follicle wall or within the CL along with ovarian venous and jugular catheters to collect simultaneous, real-time information on the ovarian and systemic changes in the secretion of factors regulating vascular function. Based on the results obtained from *in vivo* experiments, it was proposed that a physiological relevant "cross-talk" between the vascular components (endothelial cells) and steroidogenic cells occur in the bovine ovary particularly during ovulation, CL formation and regression.

Key words : Bovine, Ovary, Follicle, Corpus luteum, Blood flow

Hemodynamic changes are involved in the cyclic remodeling of the ovarian tissue that occurs during follicular growth, ovulation, and corpus luteum (CL) formation and regression (Collins et al., 1991; Brannstrom et al., 1998; Acosta et al., 2002). However, the dynamics of these events are not fully understood. Color Doppler ultrasonography is a useful, noninvasive tool for evaluating vascular function, allowing a visual observation of the blood flow in a delimited area in the wall of preovulatory follicles (Brannstrom et al., 1998) or within the CL (Miyazaki et al., 1998; Acosta et al., 2002). Since this imaging results in reproducible and quantitative measurements of local blood flow (Collins et al., 1991; Brannstrom et al., 1998; Acosta et al., 2002), a color Doppler ultrasonography was used to assess the blood flow and related changes in the vasculature taking place in the follicle wall and within the CL during specific physiological events such as ovulation, CL development and CL regression in cows.

Local changes in blood flow within the ovary are closely related to changes in the biosynthesis of prostaglandins (PGs) and steroids (Murdoch et al., 1986; Acosta et al., 2002). Angiotensin II (Ang II), endothelin -1 (ET-1), and atrial natriuretic peptide (ANP) are vasoactive peptides produced by the vascular component that modulate vascular tonus in the systemic circulation. Moreover, these vasoactive peptides have been shown to modify the synthesis and secretion of hormones produced in ovarian cells in an autocrine/paracrine manner (Kamada et al., 1992; Yoshimura et al., 1993; Johnson et al., 1994; Tedeschi et al., 1994; Acosta et al., 1998). The high concentrations of Ang II, ET-1 and ANP in the different follicular compartment (Kim et al., 1989; Gutkowska et al., 1993; Hagemann et al., 1994b; Acosta et al., 1998; Karam et al., 1999), the presence of their specific receptors (Saheki et al., 1989; Brunswig-Spickenheier and Mukhopadhyay, 1992; Tedeschi et al., 1994; Nielsen et al., 1995), and the cyclic variation in their ovarian activities during the estrous cycle (Kim et al., 1992; Hagemann et al., 1994a; Ohtani et al., 1998; Ohtani et al., 2001) suggest important roles for these vasoactive peptides in ovarian physiology.

Due to a lack of information on the local release of vasoactive peptides together with steroid hormones and PGs in the ovarian microenvironment, the capillary membranes (0.2 mm diameter and 5-10 mm length) of a microdialysis system (MDS) were implanted into the follicle wall and within the CL *in vivo*.

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Ovarian venous (OV) and jugular (JV) catheters were also inserted to collect simultaneous, real-time information on ovarian and systemic changes in the secretion of factors regulating vascular function.

Materials and Methods

Animals and Ultrasound Scanning

Normal cycling Holstein cows at the early luteal, mid luteal or preovulatory stage of the estrous cycle were used for the Doppler study. Five hundred micrograms of a $PGF_{2\alpha}$ analogue (cloprostenol [estrumate]; Sumitomo Pharm. Co., Osaka, Japan) was injected intramuscularly to induce luteolysis and ovulation. To evaluate changes in the blood flow within the CL in response to a $PGF_{2\alpha}$, the cows were treated on Day 4 (early CL) or on Days 10 to 12 (mid CL) of the estrous cycle. Ultrasonographic examinations were carried out just before PG injection (0 h) and then at 0.5, 1, 2, 4, 8, 12, 24, and 48 h after the injection. Blood samples were collected at each of these times to determine the circulating concentrations of progesterone.

The preovulatory follicle and the CL were examined by transrectal ultrasonography using an ultrasound scanner (Aloka SSD-1700, Mitaka, Tokyo, Japan) equipped with a 7.5 MHz convex transducer. During each ultrasonographic examination the volume (V) of the CL and diameter of the follicle were determined. After morphological evaluation, the flow mode was activated for blood flow mapping. Color signals were used to generate images in which blood flow was detectable in the follicle wall or within the CL. Using these images, the blood flow was evaluated in each ovarian structure. The sectional area (SA) was calculated by the following equation: $SA = \pi/4^{\times} (SD)^2$, where SD is the sectional diameter. The ratio of the colored area in the image obtained in a vertical plane at the maximum diameter of the follicle or the CL was used as a quantitative index to express the changes in the blood flow within the CL.

Blood flow velocity waves forms were recorded during 3 cardiac cycles to determine the time averaged maximum velocity (TAMXV) by placing the sample volume (Doppler gate) across the main vessel and switching on the pulsed Doppler mode. The pulsed Doppler sample volume was set at a 1-mm width. All scans were performed at a pulse repetition frequency of 6 Hz. Identical color gain settings were used for all scanning. The angle of insertion was adjusted to obtain the maximum color intensity. Scan records (images) were stored on a MO disk drive for a personal computer (Macintosh) and then viewed on the monitor of the screen. The colored area was selected and changed to a black and white image using Adobe Photoshop 5.5 software. The same image was used to calculate the area of the CL and the colored area was quantified using the NIH Image program (Version 1.62) developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image.

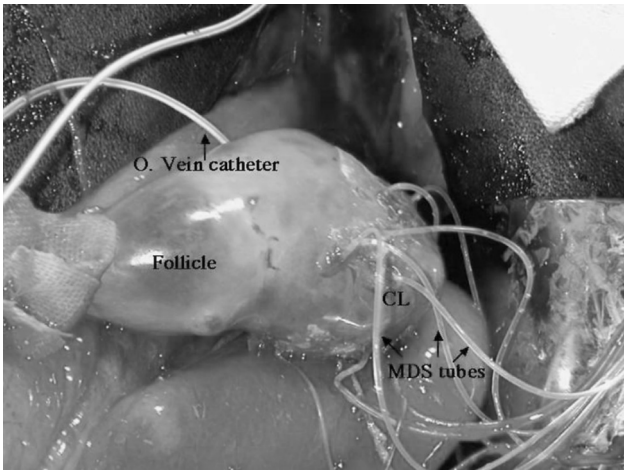


Fig. 1 Illustration of the tubes of the Microdialysis System (MDS) implanted within the corpus luteum and a catheter inserted in the ovary vein for sample collection.

Implantation of the MDS capillaries into the ovary

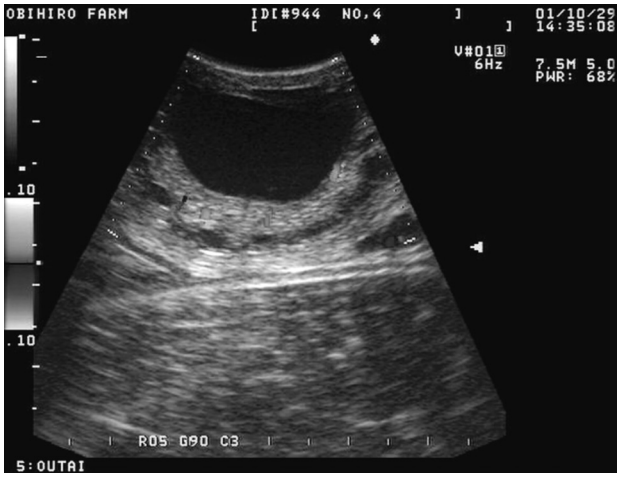
The MDS for bovine mature follicles or CL was based on the method developed for porcine follicles *in vivo* (Einspanier et al 1991), and the same system was applied previously to the bovine CL *in vivo* (Othani et al 1998) with some modifications. The MDS capillary membranes were implanted in the theca layer of follicles or within the CL. The implant was done via a lateral laparotomy under epidural anesthesia as described previously (Acosta et al 1998; Othani et al 1998). Basically, the dialysis capillary membranes (Fresenius SPS 900 Hollow Fibers, cutoff MW=1,000 kDa, 0.2 mm diameter, 5 mm long; Fresenius AG, St. Wendel, Germany) were implanted into the lateral side of the follicular wall using a 25-gauge hypodermic needle. Both ends of the capillary were glued to a 25-cm-long piece of silicone elastomer tubing (i.d. 0.3 mm) and connected to the MDS (Fig.1). The tubing was fixed on the surface of the follicular wall by Histoacryl blau (B. Braun-Dexon GmbH, Spangenberg, Germany), and the dialysis pieces with silicone tubing were connected to Teflon tubing that lead to the outside of the abdomen. The exteriorized bundle of afferent and efferent Teflon tubing was fixed on the back of the cow. One end of the MDS was con-

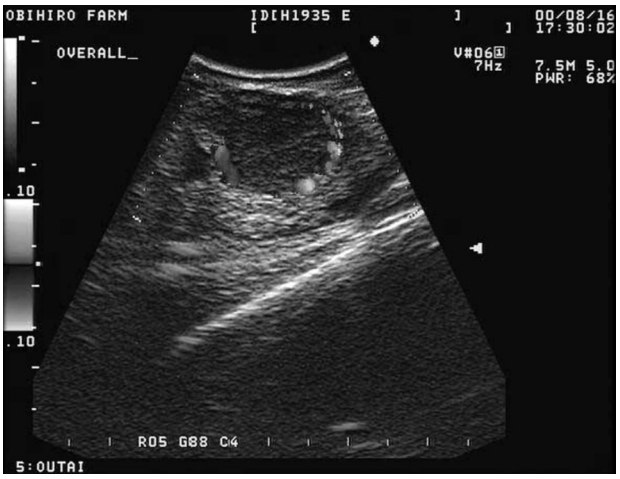
Preovulatory Follicle

Early CL

a)

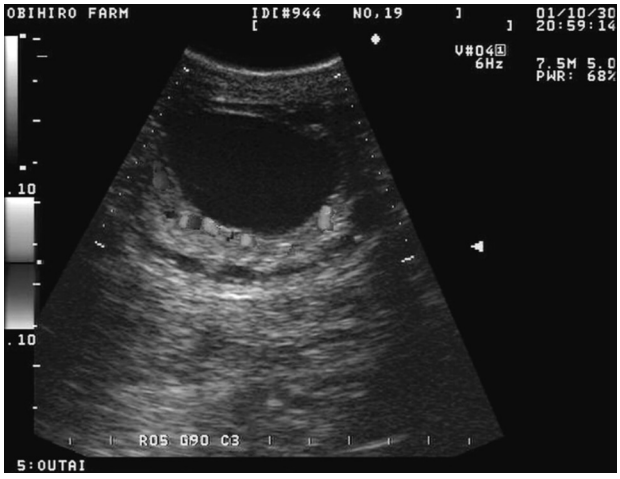
b)

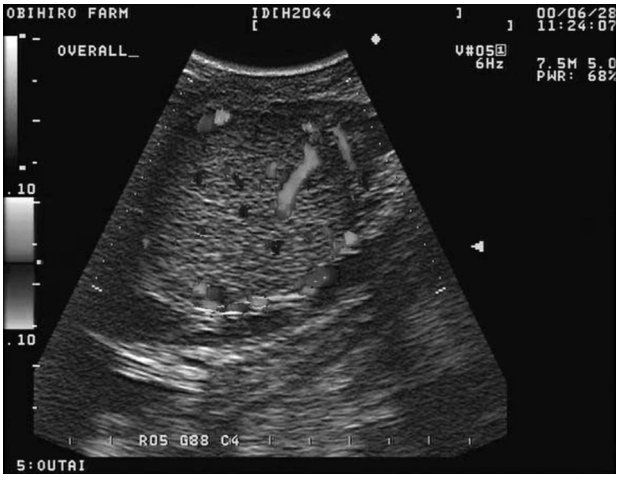




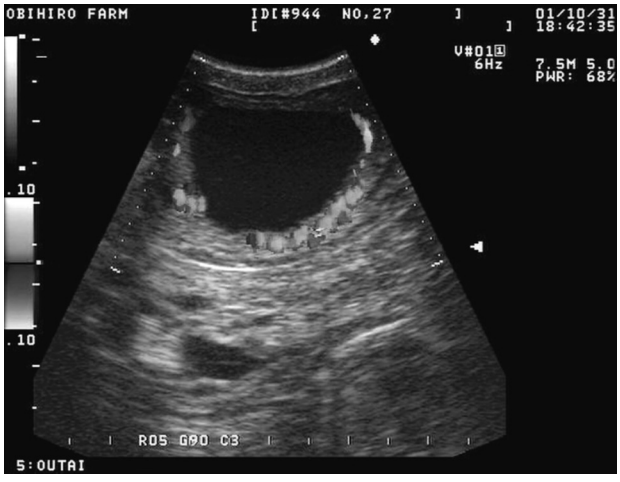
−24h

Mid CL





onset of LH surge (Oh)



24h

a) Detectable blood flow area in the preovulatory folliele wall (left panels) at −24h (top), Oh (middle) and 24h (bottom) after the onset of LH surge.

b) Detctaqble blood Floe area within the early and midcycle corpus luteum (right panels).

nected to a multiple-line peristaltic pump, and the other was connected to a multiple-line fraction collector. The MDS was continuously perfused with Ringer's solution at a flow rate of 2.5 ml/h throughout the experiments, and the fractions of the perfusates were collected at intervals of 4 h, thus allowing the determination of the local secretory changes during the process of ovulation, new CL formation and regression.

At the surgery an 18-gauge catheter (Medicut Catheter Kit; Argyle Co., Japan Sherwood, Tokyo, Japan) was inserted into the ovarian vein ipsilateral to the implanted MDS and sutured. JVP and OVP for determination of peptides, PGF 2_{α}, and steroid hormones were collected from the start of the experiment into sterile 10 ml tubes containing 200 μl of a stabilizer solution (0.3 M EDTA, 1% acid acetyl salicylic, pH 7.4). All tubes were immediately chilled in ice water for 10 min, centrifuged at $\times$2,000 g for 10 min at 4℃, and the obtained plasma was frozen at −30℃ until further analysis.

Hormone concentrations in the samples collected were determined by enzyme immunoassays after extraction procedure.

Results and Discussion

Preovulatory follicle

The Doppler studies provided visual evidence for time-related changes in blood flow within the preovulatory follicle wall of cows around the time of ovulation. The image of the preovulatory follicles revealed that blood flow area and velocity (time averaged-maximum velocity; TAMXV) were temporally correlated with an increase in plasma concentration of estradiol and the LH-surge (Fig. 2 and Fig. 3). These findings indicate that a functional relationship between blood flow in the follicular wall and plasma level of estradiol and LH exists during the periovulatory period.

The first detectable increase in plasma estradiol was coincident with an increase in vascularization (blood flow area) in the follicle wall (Fig. 3). This phenomenon may induce an acute change in the metabolic function of follicular cells, resulting in increased production of steroids and vasoactive substances. It has been demonstrated that estrogen causes a rapid dilation of blood vessels by activating endothelial nitric oxide synthase (eNOS). In studies of intact artery endothelial cells of sheep, estradiol caused acute (five-minute) activation of eNOS that was fully inhibited by concomitant treatment with estrogen receptor $(ER)_{\alpha}$ antagonists, suggesting that the short-term effects of estrogen are mediated by

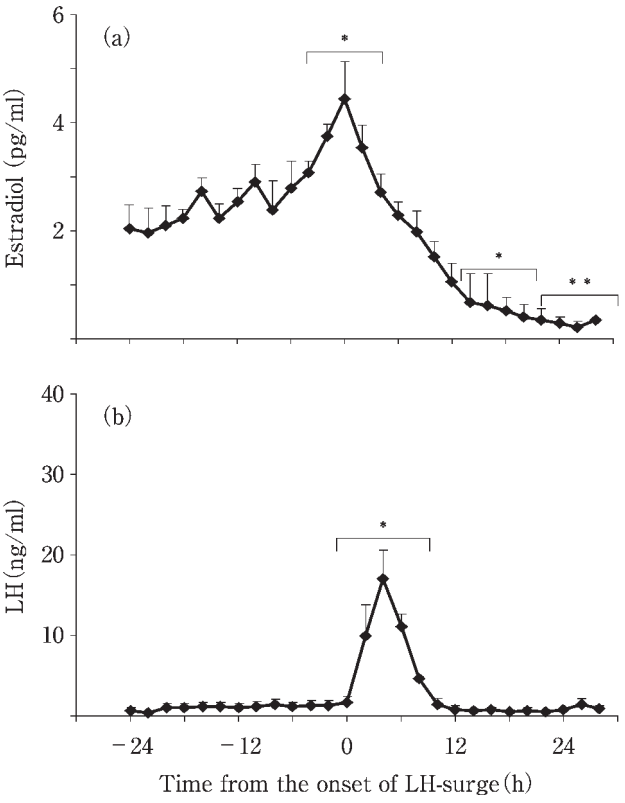


Fig. 2 Changes in the plasma concentration of estradiol (a) and LH (b) from the onset of LH surge (0 h) to ovulation. Data points show mean ± SEM for each time period (n = 5 cows per group).
*P < 0.05, **P < 0.01 vs. values before the onset of LH-surge.

ER_{α} (Chen et al., 1998). This local vasodilatory substance acts directly in the regulation of the blood flow in the ovary. The increase in the blood flow to the follicular cells in the preovulatory follicle wall may increase the supply of gonadotrophins, nutrients, hormonal substrates and other blood components necessary for ovulation.

The blood flow in the apex of ovulatory follicle decreases while it increases at the base of the follicle (Brannstrom et al., 1998) facilitating follicular rupture. However, the mechanisms of LH-induced hyperemia remain unknown. It was postulated a mediatory role of vasoactive peptides released during LH stimulation in the bovine preovulatory follicle (Acosta et al., 1999). Vasoactive peptides have been demonstrated to play important roles in the ovulation process as well as early luteal development, through modulating the local secretion of PGs and steroid hormones (Acosta et al., 1999; Acosta et al., 2000; Kobayashi et al., 2002). One of the main points for understanding the roles of vasoactive peptides in

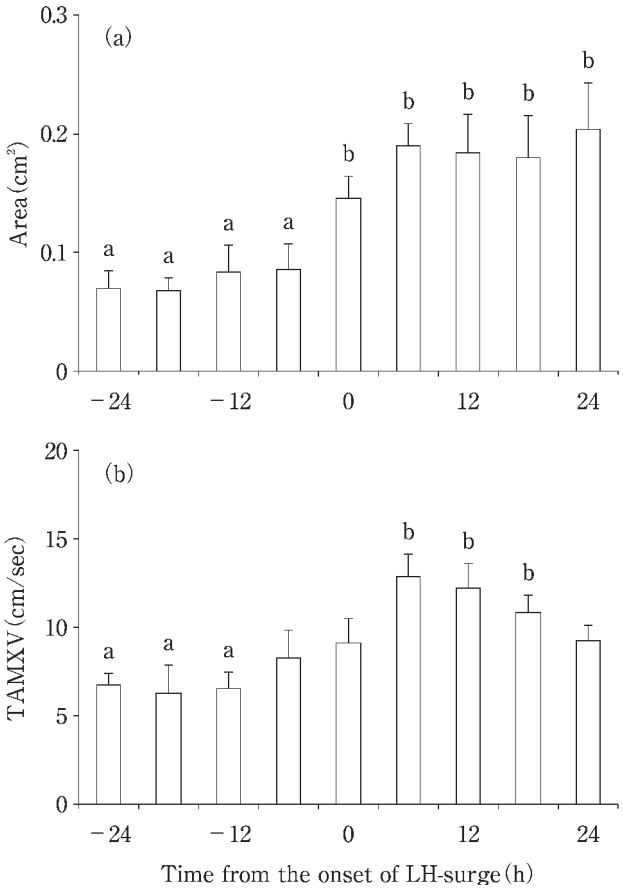


Fig. 3 Changes in the area (a) of and the corresponding Doppler-derived time-averaged maximum velocity (TAMXV; b). The TAMXV was measured in the base of the follicle with color Doppler ultrasonography. Data shows mean ± SEM for each time period (n = 5 cows per group). [a,b]values with different letters are significantly different ($P < 0.05$).

ovarian physiology is to know the changing profile of local blood flow in individual follicles and the CL at specific stages of the estrous cycle. Therefore, color Doppler studies were carried out to characterize the real-time changes in the blood flow within the follicle wall during the last stage of follicular maturation associated with the LH-surge, ovulation, and new CL development in cows.

Ang-II, ET-1 and ANP have the capacity to induce local vascular changes in different organs and tissues. Therefore the main effects of these peptides on the ovulatory process are very likely involved with control of follicular blood flow. The facts that ET-1 stimulated the ANP release, and ANP stimulated Ang II release, indicate that these peptides act in a local cascade-like reaction. This cascade might mediate the LH action to accelerate PGs production during the preovulatory period. Interestingly though, Ang II directly inhibits the release of ANP from the theca layer of the bovine mature follicle *in vitro*.

The complex structural, secretory and other functional changes that take place in the ovary around the time of ovulation are closely associated with local changes in the blood flow within the wall of the preovulatory follicle. Taken together, the above evidences suggest that interactions among vasoactive peptide, PGs and steroids play key roles in the LH-triggered ovulatory cascade in the bovine preovulatory follicle.

Differences in the vascular response to a Prostaglandin $F_{2\alpha}$ injection between the early and mid-CL

After ovulation, the new CL rapidly develops from the wall of the ruptured follicle within a few days. This growing period is characterized by active angiogenesis and a parallel increase in progesterone secretion (Fig. 4a).

One of the main luteolytic actions of $PGF_{2\alpha}$ is to decrease ovarian blood flow (Knickerbocker et al., 1988). However, before Day 5 of the estrous cycle, the CL is refractory to the luteolytic action of $PGF_{2\alpha}$ (Tsai et al., 1998). A color Doppler study was performed to compare the real-time changes in intraluteal blood flow after $PGF_{2\alpha}$ injection at the early (Day 4) and middle (Day 10) stages of the estrous cycle in the cow. A clear and acute increase in the blood flow within the midcycle CL following injection of a luteolytic dose of $PGF_{2\alpha}$ analogue was observed. In contrast, these changes were not detected in the early CL in which the plasma progesterone concentration (Fig. 4a), CL volume (Fig. 4c), and TAMXV (Fig. 5c) increased from Day 4 to Day 6 in spite of the $PGF_{2\alpha}$ injection. The results suggest that an initial increase in intraluteal blood flow induced by PG injection in the midcycle CL may trigger the initiation of the luteolytic cascade in the cow.

The exact mechanism and physiological relevance of the initial increase in the blood flow remain unknown. The transitory hyperemic reaction appears to involve the dilatation of arterioles, precapillary sphincters, and post capillary venules. PGs modulate vascular resistance in most of the vascular beds of the body, including those involved in uterine and ovarian circulation (Ford et al., 1977; Valentin et al., 1995). It was recently found that an injection of a $PGF_{2\alpha}$ analogue induced an acute release of PGE_2 and $PGF_{2\alpha}$ from the midcycle CL in the cow (Hayashi et al., 2003). Moreover, the same PG injection acutely induced the expression of cyclooxygenase (COX)-2 enzyme from 1

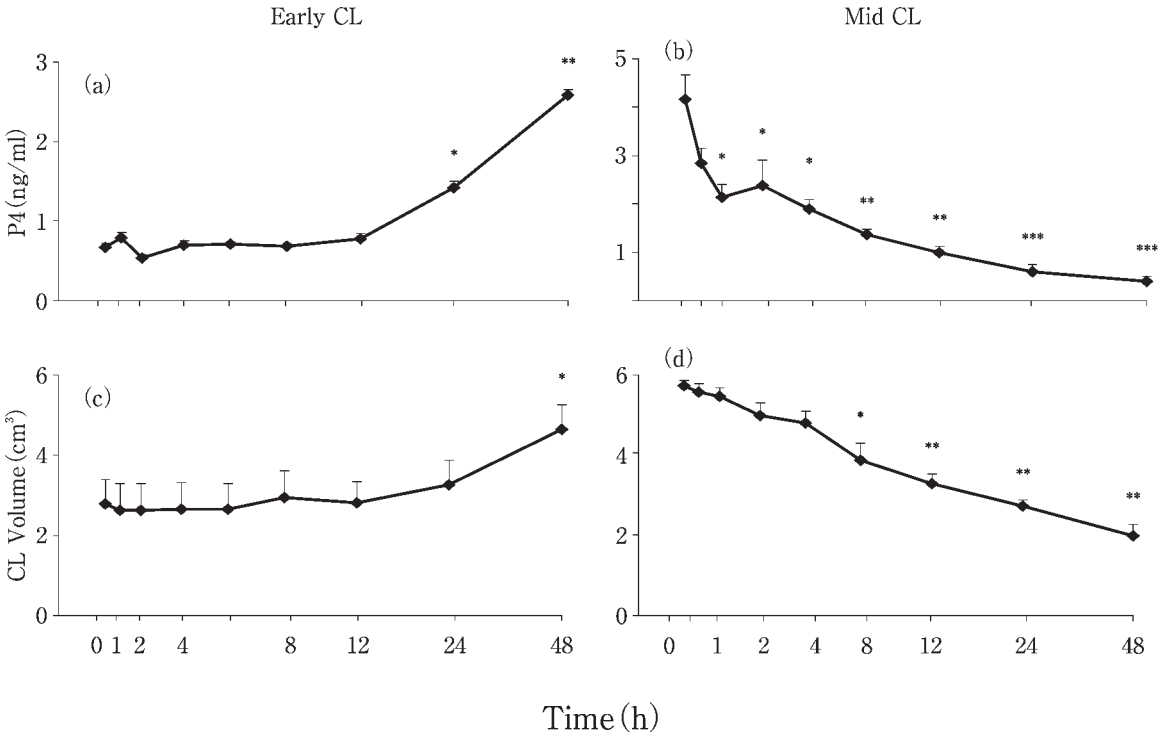


Fig. 4 Changes in the plasma concentration of progesterone (a, b) and the volume of the corpus luteum (c, d) at early (left) and middle (right) stages of the estrous cycle, relative to the injection of a luteolytic dose of PGF_2 xay (0 h). Data points show mean ± SEM for each time period (n=5 cows/group).
*P < 0.05, **P < 0.01, ***P < 0.001 vs. values before PGF_2 xay injection.

h to 4 h postinjection in the cow (Tsai et al., 1998; Levy et al., 2000; Hayashi et al., 2003) and the ewe (Tsai et al., 1997), but did not affect the COX-2 expression in the early CL (Valentin et al., 1995). These findings suggest that PG synthesis from arachidonic acid and the enzymatic action of COX-2, including the synthesis of vasodilatory PGI 2, may cause this acute increase in blood flow within the midcycle CL.

Nitric oxide (NO) is another local vasodilatory substance that may play a direct luteolytic role in the regressing CL (Jaroszewski and Hansel, 2000). It has been demonstrated that PGs modulate luteal NO synthase (NOS) activity and progesterone production, depending on the stage of the CL. $PGF_{2\alpha}$ caused a 2.5-fold increase of NOS activity and a marked decrease in progesterone production on Day 9 in rabbit CLs (Boiti et al., 2000). NO also acutely inhibited progesterone release from Day 9 CLs of pseudopregnant rabbits (Gobbetti et al., 1999).

In cows with a mid-CL, a significant decrease in plasma P_4 concentration was first observed 30 min to 1 h after PG injection, and P_4 concentration decreased further over the time (Fig. 4b). The CL volume (Fig. 4d) and TAMXV (Fig. 5d) remained relatively unchanged until 8 h. These results demonstrate that PGF 2_{α}-induced decrease in plasma P concentration occurs before the occurrence of a significant decrease in the volume of the CL and in luteal blood flow. In addition, the reduction in luteal blood supply at 8 h after $PGF_{2\alpha}$ injection was coincident with the time of the initiation of structural luteolysis, which was reflected in the first significant decrease in the volume of the CL (Fig. 4d).

Vascular changes during luteolysis included an initial acute increase in the blood flow expressed by an increase in the relative color area of the midcycle CL. However, such a change was not observed following the injection of $PGF_{2\alpha}$ in the early CL. The absence of this change within the early CL, in which luteolysis did not occur, and the progressive vascular changes in the midcycle CL, in which luteolysis did occur, suggest that these vascular alterations are stage and PG dependent and are necessary for the induction of the local release of ET-1 (Ohtani et al., 1998; Levy et al., 2000) and Ang II (Ohtani et al., 2001), which further induce a decrease in the blood supply (flow) as a result of vasoconstriction. The initial acute increase in intraluteal blood flow at 0.5–2 h after $PGF_{2\alpha}$ injection (Fig. 5b) may be crucial for stimulating luteal endothelial cells to produce and release vasoactive sub-

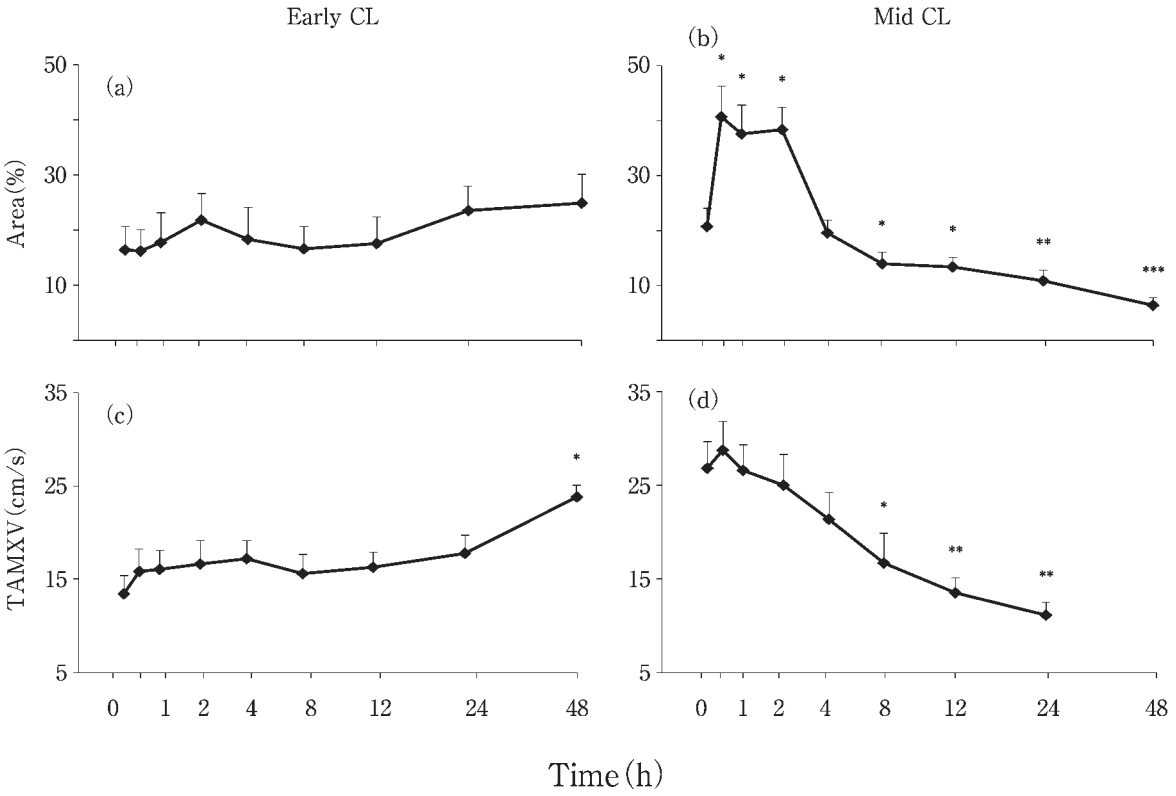


Fig. 5 Relative changes in the area of detectable blood flow in the early (a) and middle cycle (b) CL expressed as the percentage of the area of the CL. The TAMXV (c, d) was measured in the base of the CL with color Doppler ultrasonography. Data points show mean ± SEM of each time period (n = 5 cows/group).
*P < 0.05, **P < 0.01, ***P < 0.001 vs. values before PGF_2 xay injection.

stances necessary to trigger the cascade of changes leading to luteolysis. Taken together, these findings indicate that $PGF_{2\alpha}$ induces an acute blood flow increase followed by a decrease within the midcycle CL but not in the early CL. This transitory increase may trigger the luteolytic cascade. The lack of intraluteal vascular response to PG injection in the early CL appears to be directly correlated with the ability to be resistant to PG.

Conclusions

The increases in the follicular blood flow around the time of ovulation and within the CL at initial stage of regression are associated with a drastic increase in the local secretion of prostaglandins and vasoactive peptides. Color Doppler Ultrasonography and Microdialysis System represent useful approach to investigate these functional changes in the ovarian structures. The acute changes in the blood flow may trigger the cascades of the final step of ovulation and the initial step of CL regression, respectively. The overall results indicate that a functional "cross-talk" between the vascular components (endothelial cells) and steroidogenic cells occur in the bovine ovary.

Acknowledgments

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ウシ卵巣における血管系の役割
―― 卵胞，黄体における血流およびホルモン分泌の変化 ――

アコスタ　アヤラ　トーマス
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カラードップラー超音波断層診断装置は，直腸を介して卵巣内の構造変化および血流変化を評価することができ，排卵，黄体形成，黄体退行などの卵巣生理現象の観察に有効である．私は，ウシにおいてカラードップラー超音波断層診断装置を用いて卵胞壁および黄体内の血流変化について検討するとともに，微透析システム（MDS；microdialysis system）を卵胞壁および黄体内に装着し，局所的な血管作動性物質，ステロイドホルモンおよびプロスタグランディン類の分泌を調べた．また，卵巣静脈および頚静脈より血液を経時的に採取し，血管機能を調節する因子の経時的変化についても検討した．これらの成果から，ウシの卵巣生理現象（特に，排卵，黄体形成，および黄体退行）において，卵巣内の血管内皮細胞とステロイド産生細胞（卵胞内膜細胞，顆粒層細胞および黄体細胞）間にクロストーク（相互調節作用）の存在することが示唆された．