Association of luteal blood flow with follicular size, serum estrogen and progesterone concentrations, and the inducibility of luteolysis by PGF$_{2\alpha}$ in dairy cows

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**A B S T R A C T**

The aim of this study was to investigate the compatibility of the visual evaluation result of the blood flow characteristics and the blood flow measurements of the CL and the predictability of the responses given by corpora lutea with varying levels of blood flow to an induction of luteolysis by a PGF$_{2\alpha}$ injection and to determine the possibility of increase in serum estrogen and progesterone concentrations in parallel with increased luteal blood flow (LBF). The cows, bearing a CL ($n = 60$; postpartum 35 days), were injected with PGF$_{2\alpha}$ and were monitored for signs of estrous following the first injection. The cows, which did not show estrous signs, were examined for the presence of a CL on Day 14, whereas those that showed signs of estrous were examined on Day 10 following the onset of estrous. The level of LBF was visually graded as $+$ (low; G1), $++$ (medium; GII), $+++$(high; GIII), and $++++$ (very high; GIV). Immediately after the examination of LBFs, a second intramuscular injection of PGF$_{2\alpha}$ was injected. In the cows, which were determined to be in estrous, the diameter of the Graafian follicles was measured by B-mode ultrasonography. Subsequently, these animals were artificially inseminated. The animals, which did not show estrous after the second injection, were examined as previously described and monitored for signs of estrous. A strong correlation ($r = 0.654; P < 0.001$) was determined to exist between the results of the visual examination of the images and the results obtained for the LBF area with the use of the Pixel Flux software. GIII ($0.83 \pm 0.15$ cm$^2$) and GIV ($1.03 \pm 0.48$ cm$^2$) were found to differ from GI ($0.47 \pm 0.23$ cm$^2$) and GII ($0.51 \pm 0.12$ cm$^2$) for the size of the LBF ($P < 0.001$). Serum progesterone levels in groups (GI, GII, GIII, and GIV) were determined to be 4.44 $\pm$ 2.42 ng/mL, 6.03 $\pm$ 2.37 ng/mL, 7.01 $\pm$ 2.94 ng/mL, and 7.17 $\pm$ 1.69 ng/mL, respectively. The comparative evaluation of the study groups showed that the groups did not statistically differ for the period between PGF$_{2\alpha}$ injection and the onset of estrous, mean Graafian follicle size and estrogen levels. No direct correlation existed between these reproductive parameters and LBF.

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1. Introduction

The CL, which has a lifespan of 17 to 18 days, is an endocrine structure that is involved in the establishment and maintenance of pregnancy by producing progesterone...
Among all body tissues, it is the CL, which receives the highest level of blood flow in proportion to its size [2]. The vasculature of the CL supplies the luteal tissues and enables not only the transport of the hormones and hormonal substances required for the secretion of progesterone (P4) but also the release of secreted progesterone into the systemic blood circulation [3–6]. Furthermore, luteal endothelial cells secrete various vasoactive substances, including nitric oxide (NO), endothelin-1, angiotensin II (Ang II), and prostaglandins (PGs), all of which are directly involved in the regulation of P4 secretion. Therefore, the blood vessels and endothelial cells of the CL play an important role in the functionality of the CL [1].

Color Doppler ultrasonography is a noninvasive diagnostic method used for the visual observation of the blood flow within the CL and the wall of the preovulatory follicle [7], and the evaluation of the ovarian vascular function [8]. In the past 15 years, color Doppler ultrasonography has replaced invasive techniques for the monitoring of the bovine reproductive system [9]. Recently, luteal blood flow (LBF) measurement has started to be used for the determination of the functional status of the CL [8,10–14]. In previous research, LBF has been investigated throughout the estrous cycle [15], has been used for early pregnancy diagnosis [16,17] and the detection of nonpregnant dairy and beef cows at Day 20 after timed artificial insemination [18–20], and has been tested in response to different hormone treatments [14]. Some literature reports suggest that LBF increases in parallel with the increase of the size of the CL and progesterone level during the development of the CL and indicate that LBF level is strongly correlated with progesterone production [5,15,21].

Luteolysis is described as the lysis or structural death of the CL [22]. Prostaglandin F2 alpha is a luteolytic factor, which is secreted from the uterus and initiates luteolysis in the CL. It causes a striking decrease in the progesterone level and reduces the size of the CL [1]. It has been reported that PGF2α receptors are mostly located in the endothelial cells and large blood vessels situated in the periphery of the CL and are found to a less extent in the small blood vessels in the center of the CL. Prostaglandin F2 alpha acutely stimulates endothelial NO synthase and increases LBF in the periphery of the CL. Nitric oxide is directly involved in the regression of the CL, owing to its vasodilator effect of the arterioles [23]. Increasing the LBF, accompanied by immune cell infiltration, increase in chemokines, and expression of major histocompatibility complex molecules, causes the functional luteolysis. But then, PGF2α directly increases endothelin-1 and Ang II secretion from microvessels within the CL. These vasoactive peptides suppress P4 secretion and induce chronic vasoconstriction of the arterioles of the CL. Structural regression of the CL is indicated by a gradual reduction in CL size and ensure luteolysis [24]. The prolongation of the luteolysis results in the prolonged dominance of the preovulatory follicle [25]. The permanence of the dominant follicle alters the environmental factors that influence oocyte development and causes embryonic degeneration, which eventually leads to reduced fertility [26]. Therefore, it is suggested that cows with higher LBF levels may display a more evident estrous response to PGF2α injection. The aim of this study was to investigate the compatibility of the visual evaluation result of the blood flow characteristics and the blood flow measurements of the CL and to determine the responses of corpora lutea (different proportion of luteal area with blood flow signals) to PGF2α treatment and the correlation of LBF level with follicle size and estrous and progesterone levels.

2. Materials and methods

This study was conducted after obtaining approval from the Animal Experiments Local Ethics Committee of Selcuk University (SUVFEK—Submission: 19.02.2013/004).

2.1. Animals

The study material comprised of 60 Holstein cattle, which were raised in a semi-closed system in a private holding and fed on a total mixed ration (corn silage, dry alfalfa, dry vetch-triticale, wheat hay, corn flex, soya oilcake, and pellet feed for dairy cows). The average parity of the cows (aged 3–11 years) was 2.53 ± 0.22. Immediately after the first PGF2α injection, body condition scores were assessed as described by Edmonson et al. [27] (scale 1–5, in increments of 0.25). Although the body condition scores of the animals ranged between 2.50 and 3.75, their average milk yield during lactation was 27.5 ± 5.5 L.

2.2. Assessment of luteal blood flow

Clinically healthy animals, which were determined to have a CL by transrectal palpation and ultrasonographic examination on Day 35 postpartum, were injected with PGF2α (5 mL, 5 mg/mL, dinoprost, Enzaprost, CEVA-DIF, Turkey) and were monitored for signs of estrous following this first injection. The animals, which did not present with any signs of estrus, were examined on Day 14 after the first PGF2α injection, whereas the animals, which displayed signs of estrus, were examined on Day 10 after the onset of estrus for the presence of a CL. B-mode and power Doppler mode ultrasonographic examinations and image collection were performed as described previously [11,28]. The CL was identified by B-mode ultrasonography (10-MHz frequency, LOGIQ Book XP, General Electric Healthcare, Solingen, Germany), and its image was frozen at the maximum cross-sectional area and stored for further offline measurements. Later, the images were examined by the power Doppler mode (gain: 19.5, pulse repetition frequency: 0.5 KHz, Doppler frequency: 5 MHz) of the same device equipped with linear probe for the imaging of the blood flow to the CL. Color LBF mapping in various transverse sections was conducted using the power Doppler mode. To minimize the variations in recording, the settings of the power Doppler system were fixed and used for all examinations. At least five images without flash artifacts and with the maximum number of colored areas were stored in the memory of the ultrasound device in Digital Imaging and Communications in Medicine (DICOM) format. The analysis of the stored Doppler images (five images of the blood flow area of the CL) was performed using an image processing software (Pixel Flux, Version 1.0, S. Kaya et al. / Theriogenology 87 (2017) 167–172.
Chameleon-Software, Leipzig, Germany). For this purpose, the entire luteal structure and its blood flow area were chosen as the region of interest, and the colored area within this region of interest was calculated. The averages of the five images were used for further evaluation of LBF. The LBF quantitative data obtained with the use of the image processing software were compared with the levels of LBF determined by visual examination.

The level of blood flow to the CL was visually graded as + (low; GI), ++ (medium; GII), +++ (high; GIII), and ++++ (very high; GIV). Visual LBF grading was performed on the basis of the proportion of the luteal area filled by the blood flow, in comparison to the total luteal area (Fig. 1). All ultrasonographic examinations were conducted by the same operator.

Immediately after the examination of LBFs, a second intramuscular injection of PGF2α (5 mL, 5 mg/mL, dinoprost, Enzaprost, CEVA-DIF) was injected. Seven days after second PGF2α injection, the animals were monitored three times a day, each time for half an hour, for signs of estrous. The date and time of estrous onset were recorded. The diameter of the Graafian follicles was measured by B-mode ultrasonography. The cows, which were determined to have a Graafian follicle 8 to 12 hours after estrous, were sampled for blood from the coccygeal vein and then artificially inseminated. The animals, presenting with no signs of estrous, were examined as previously described, and 14 days after the second PGF2α injection (for the measurement of the diameter of the CL and the imaging of the blood flow area of the CL by Doppler ultrasonography), they were also monitored for signs of estrous. Of these animals, those that were determined to be in estrous were artificially inseminated. The artificially inseminated animals were examined for pregnancy on Days 35 and 55 after insemination by B-mode ultrasonography (Fig. 2).

2.3. Hormone analyses

Immediately after the power Doppler ultrasonographic examination of each animal, blood samples were collected from the coccygeal vein. The blood samples were centrifuged at 1200 × g for 10 minutes, and the extracted sera were stored at −20 °C until the hormone assays were conducted. The estrogen and progesterone levels in the serum samples were measured using the electrochemiluminescence immunoassay (Test Roche E170) as described by Ay et al. [28] and Kaya et al. [29]. The lower detection limit of estrogen and progesterone was 5.0 pg/mL and 0.03 ng/mL, respectively. The intra-assay and inter-assay coefficients of variation of estrogen and progesterone were 1.4%, 2.6% and 0.7%, 1.8%, respectively.

2.4. Statistical evaluation

The statistical evaluation of the results was performed using SPSS (SPSS 16, IL, USA). Luteal blood flow, luteal size (LS), period between PGF2α injection and the onset of estrous (PG-E), Graafian follicle size (FS), P4 and estrogen levels (E2) in each group were tested for normality using the Shapiro–Wilk test. The comparison of the study groups for LBF, LS, PG-E, FS serum, and P4 and estrogen levels was performed by the Kruskal–Wallis test. The correlation between the LBF level, LS, and blood serum P4 level was assessed by Spearman’s rho correlation test for the study groups. The correlation between the results of the visual examination of the power Doppler images and the results obtained for the LBF area with the use of the Pixel Flux software was assessed by a polyserial correlation test. Results are expressed as mean ± standard deviation of the mean (X ± SD). Differences and correlations with P-values less than 0.05 were considered to be significant.

3. Results

In this study, 300 images of 60 corpora lutea were assessed both visually and with the aid of the Pixel Flux software. According to the visual evaluation of the images acquired by the power Doppler ultrasonographic examination of the CL, the level of LBF was graded as low in eight cows (GI, +), medium in 13 cows (GII, ++), high in 23 cows (GIII, +++), and very high in 16 cows (GIV, ++++) (Fig. 1). The comparison of the results of the LBF area value measurements performed using the Pixel Flux software with the LBF levels determined by visual examination (+, 1, 2, 3, 4) demonstrated that the results achieved with both assessment methods were strongly correlated with each other (r = 0.654; P < 0.001). In parallel with the increase in LBF level, which was visually graded from +1 to +4, it was determined that the luteal values measured increased from 0.47 cm² to 1.03 cm².

The size of the LBF area measured in GI was ascertained to be smaller than that measured in GII, but this difference was found to be statistically insignificant. On the other hand, the size of the LBF in GI and GII was determined to be smaller than the sizes measured in GIII and GIV (P < 0.001). Similarly, the animals included in GIII and GIV were found

![Fig. 1](https://example.com/fig1.png) The visual grading of the level of blood flow to the CL (A: Low, B: Medium, C: High, D: Very high).
to differ from each other for the size of the LBF ($P < 0.001$). Software measurements demonstrated that the LS did not significantly differ among the groups. It was determined that serum P4 levels had increased in parallel with the increase determined in LBF level by visual evaluation, yet this difference was found to be insignificant. The comparative evaluation of the study groups on the basis of visual examination demonstrated that no statistically significant difference existed between the study groups for PG-E, mean of FS, E2, or pregnancy rate (Table 1).

The comparison of the alterations observed in all animals for the size of the LBF area and P4 levels showed that the two parameters were significantly correlated with each other ($r = 0.318$; $P = 0.02$). However, it was observed that neither LBF and LS nor LS and P4 level were significantly correlated.

### 4. Discussion

The determination of the physiological function of the CL plays a significant role in increasing the rate of success of synchronization protocols, assessing fertility, and selecting the recipient dam for embryo transfer [30,31]. In the past few years, color Doppler ultrasonography has become one of the major techniques used for the determination of the LBF area and rate of blood flow to assess luteal function [30]. In previous research, the colored area determined by Doppler ultrasonography comprised between 20% and 55% of the CL [10,13,19]. In the present study, the LBF area was determined to constitute 8.0% to 25.5% of the CL. These differences between studies for the size of the colored area (%) are thought to have resulted from the use of devices with different sensitivity. Besides, vasoactive factors, including the angiogenesis and vascular endothelial growth factors and basic fibroblast growth factor, are synthesized by the CL and are involved in both cell proliferation and luteal function [1]. It is considered that alterations in LBF may be related to the activation of these factors. In previous research, the visual evaluation of the blood flow level to the CL has been reported as an efficient and rapid method [18,20]. In agreement with previous studies, the present study demonstrated a significant correlation between the results of the visual grading of LBF and pixel values ($r = 0.654$; $P < 0.001$).

As LBF enables the transport of steroid precursors and gonadotropins to the luteal cells [15], it is considered essential to the regulation of luteal function [5,9]. Several studies have demonstrated that the correlation between LBF and P4 level varies. Although some researchers suggest that LBF and P4 levels are strongly correlated with each other [8,15,32,33], some other researchers indicate that the progesterone level is independent from LBF [34,35]. The correlation between LBF and P4 level has been reported to be stronger than the correlation between LBF and luteal diameter [15]. In the present study, LBF increase and P4 increase were found to be significantly correlated with each other. Furthermore, previous research has shown that although progesterone level and decrease in ovarian blood

**Table 1**

Comparison of the serum progesterone and estrogen levels, period between PGF2α injection and the onset of estrous, and Pixel Flux values in the cows classified according to the visual evaluation of the Doppler images (mean ± standard deviation).

<table>
<thead>
<tr>
<th>Variables</th>
<th>GI (n = 8)</th>
<th>GII (n = 13)</th>
<th>GIII (n = 23)</th>
<th>GIV (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBF (cm²)</td>
<td>0.47 ± 0.23ᵃ</td>
<td>0.51 ± 0.12ᵃ</td>
<td>0.83 ± 0.15ᵇ</td>
<td>1.03 ± 0.48ᵇ</td>
</tr>
<tr>
<td>LS (cm²)</td>
<td>6.12 ± 1.63</td>
<td>5.80 ± 1.20</td>
<td>4.62 ± 1.12</td>
<td>5.06 ± 1.12</td>
</tr>
<tr>
<td>LBF/LS (%)</td>
<td>8.21</td>
<td>9.05</td>
<td>16.59</td>
<td>25.43</td>
</tr>
<tr>
<td>P4 (ng/mL)</td>
<td>4.44 ± 2.42</td>
<td>6.03 ± 2.37</td>
<td>7.01 ± 2.94</td>
<td>7.17 ± 1.69</td>
</tr>
<tr>
<td>PG-E (hours)</td>
<td>79.5 ± 30.61</td>
<td>65.0 ± 46.72</td>
<td>69.0 ± 31.23</td>
<td>72.5 ± 40.02</td>
</tr>
<tr>
<td>FS (cm)</td>
<td>1.72 ± 0.2</td>
<td>1.78 ± 0.2</td>
<td>1.86 ± 0.2</td>
<td>1.81 ± 0.3</td>
</tr>
<tr>
<td>E2 (pg/mL)</td>
<td>17.14 ± 6.31</td>
<td>24.29 ± 19.61</td>
<td>19.87 ± 25.12</td>
<td>16.14 ± 15.6</td>
</tr>
<tr>
<td>PR (%)</td>
<td>62.5 (5/8)</td>
<td>30.76 (4/13)</td>
<td>34.78 (8/23)</td>
<td>56.3 (9/16)</td>
</tr>
<tr>
<td>PG-E (hours)</td>
<td>79.5 ± 30.61</td>
<td>65.0 ± 46.72</td>
<td>69.0 ± 31.23</td>
<td>72.5 ± 40.02</td>
</tr>
</tbody>
</table>

³ᵇᵃᵇ  = 0.001.

Abbreviations: E2, estrogen levels; FS, Graafian follicle size; GI, low LBF; GII, medium LBF; GIII, high LBF; GIV, very high LBF; LBF, luteal blood flow area; LBF/LS, ratio of the LBF to the LS; LS, luteal size; n, number of cows; P4, progesterone levels; PG-E, the time period between PGF2α injection and the onset of estrous; PR, pregnancy rate.
flow are significantly correlated with each other [36,37]. The measurement of the LBF level is a quite reliable method for the prediction of the luteal status [15]. In the present study, statistically significant differences were determined between the groups with low LBF levels (GI, GII) and the groups with high LBF levels (GIIL, GIV) (P < 0.001), yet no significant difference was detected between these groups for P4 level. The P4 levels of the groups being similar despite the strong correlation between LBF and P4 levels suggests that P4 level is an independent variable. These results show that although color Doppler ultrasonography reflects the vascular alterations in the CL, when used alone, it does not provide adequate information on the P4 level. In agreement with the results of the present study, Luttgenau et al. [35] reported that the progesterone level in the midluteal phase was independent from the LBF.

Literature reports are available on the correlation of the luteal diameter with LBF. Although Miyazaki et al. [8] have reported that CL diameter and LBF are correlated with each other, Bollwein et al. [34] and Herzog et al. [15] have suggested that no significant correlation exists between these parameters. While no significant alteration occurs in the luteal diameter during the static phase of the CL, LBF significantly increases during the same period [15]. In the present study, it was determined that the LS was smaller in the groups with a larger LBF (GIIL and GIV), yet this difference was found to be statistically insignificant (P < 0.05). Thereby, in agreement with the previous studies by Bollwein et al. [34] and Herzog et al. [15], it was determined that the luteal diameter had no effect on LBF or P4 production.

In most previous studies, the onset of estrus was detected mostly 3 to 4 days after the PGF2α injection, and the period between the prostaglandin injection and the onset of estrus was determined to range from 2 to 6 days [38–40]. Furthermore, it has been reported that the pregnancy rates achieved in animals with large follicles on the day of artificial insemination are higher [41,42]. In the present study, the onset of estrus was detected, on average, 3 to 4 days after the PGF2α injection. It was determined that, the size of the LBF area did not affect the PG-E, and did not cause an increase in the FS or the serum E2 levels. Although PGF2α does not affect the LBF level during the early luteal phase, it has been reported that PGF2α injection (within 30 minutes) significantly increases LBF levels in mature corpora lutea [10]. But, it has been demonstrated on the contrary by Shrestha et al. [43]. This study determined acute increase in LBF after PGF2α treatment (30 minutes) in early luteal phase and midluteal phase. Prostaglandin F2alpha acutely stimulates endothelial NO synthase [43]. Nitric oxide is a potent vasorelaxant and causes an increase in blood flow. Thus, NO is directly involved in the regression of the CL [44]. It is known that other factors are also involved in luteolysis. Endothelin-1 and Ang II play an important role in luteolysis [1,11,45]. Therefore, it should be borne in mind that these factors are also influential on luteolysis and that the period between the prostaglandin injection and onset of estrus not displaying any alteration with the size of the LBF could be related to these factors. All these factors affect both luteolysis and ovarian follicular development following luteolysis. Furthermore, nutrition is yet another important factor influential on follicular development, maturation, and ovulation capacity. Nutrition affects ovarian activity both directly (via GnRH secretion from the hypothalamus and gonadotropin secretion from the hypophysis) and indirectly (via the growth hormone-insulin like growth factor-insulin axis) [46]. In the present study, no difference having been determined between the groups for FS, and thus, E2 values could be attributed to these reasons.

In result, it was determined that the visual examination results of the power Doppler images were strongly correlated with the results obtained for the size of the LBFs. Likewise, the correlation between the P4 levels and LBF was significant. Furthermore, it was observed that, when compared to the cows with lower LBF levels, in the cows with high LBF levels, PGF2α injections did not alter the PG-E interval or FS and E2 levels.

Acknowledgments

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Competing interests

The authors declare that there is no conflict of interest.

References


