

—Original Article—

Luteal blood flow measured by Doppler ultrasonography during the first three weeks after artificial insemination in pregnant and non-pregnant *Bos indicus* dairy cows

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Abstract. The objective of this study was to determine if there are differences in luteal size (LS), progesterone (P4), and luteal blood flow (LBF) between pregnant and non-pregnant *Bos indicus* dairy cows during the first three weeks after insemination, and whether these parameters are related to each other. Lactating cows (n = 13) of mixed parity with a body weight of 430 ± 18 kg (mean ± SD), showing regular estrous cycle were used in the study. All cows were artificially inseminated and were classified as pregnant (embryonic heartbeat on day 30; n = 8) or non-pregnant (inter-estrus interval 17 to 21 days, n = 5). In order to compare the LS and LBF after artificial insemination, B-mode and color Doppler ultrasonography of ovaries were performed on days 4, 5, 6, 7 (first week), 8, 10, 12, 14, (second week), and 16, 17, 18, 19, 20, 21 (third week) in pregnant and non-pregnant cows. Results revealed that the mean LBF was consistently higher (P < 0.05) during days 7 through 21 in pregnant cows than in non-pregnant cows. The mean LS was higher (P < 0.05) on days 6 and 7, and from day 17 onwards, and the mean concentration of P4 was higher (P < 0.05) on days 19, 20, and 21 in pregnant cows. In conclusion, LBF is a more sensitive parameter than LS and P4 for detection of differences in luteal function between pregnant and non-pregnant *Bos indicus* dairy cows during the first three weeks after AI.

Key words: *Bos indicus* dairy cows, Doppler ultrasound, Luteal blood flow, Pregnancy

(J. Reprod. Dev. 65: 29–36, 2019)

B*os indicus* cows predominate in tropical and sub-tropical regions because of their adaptation to high temperatures and humidity [1], thereby having a considerable effect on the beef and dairy industries in these regions [2]. However, they exhibit lower potential for milk production than *Bos taurus* breed of cattle [3]. In order to optimize the milk production around the year, healthy cows must calve at 12–14-month intervals. *Bos indicus* cows have the potential to produce one calf per year, when maintained under favorable conditions [4]. The success of calving as a one year phenomenon relies on the early detection and timely re-insemination of non-pregnant cows [5, 6].

The most common method for early detection of pregnancy in cows is using transrectal B-mode ultrasonography 26–33 days post artificial insemination (AI) [7]. Alternatively, immunological detection

of pregnancy-specific substances in maternal serum, 28–35 days post AI, has been used [8–10]. These methods detect pregnancies later than the maternal recognition of pregnancy, which occurs around days 15 to 17 [11]. As these biochemical methods are relatively complex, their field application is quite restricted. Understanding of the luteal blood flow (LBF) using Doppler ultrasonography, around day 20 post AI, has emerged as a novel tool to detect and re-inseminate the non-pregnant *Bos taurus* cows [12, 13]. Although, some information on LBF is available in Nelore cows of *Bos indicus* species [14], which have beef character, there is no information for milk breeds yet.

Color Doppler ultrasonography (CDU) has extended the scope of imaging from an anatomical to a physiological basis [15]. Initially, this technique was used to measure the real-time changes in LBF after induced [16] and spontaneous [17] luteolysis in Holstein Friesian cows. Furthermore, CDU was used to compare the LBF with luteal size (LS) and concentrations of progesterone (P4) during the estrous cycle of mares [18], Holstein cows [19], Italian Mediterranean buffaloes [20], and ewes [21, 22]. Subsequently, researchers became interested in using this technique to predict the pregnancy in crossbred beef cows [23], German Holstein cows [24], Italian Mediterranean buffaloes [25], mares [26], beef heifers [27], and Holstein Friesian

Received: July 4, 2018

Accepted: October 15, 2018

Published online in J-STAGE: November 4, 2018

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cows [28]. More recently, it was used to predict the occurrence of embryonic loss based on the uterine and ovarian vascular dynamics in dairy cows [29].

Most of the research using CDU in reproductive medicine has been carried out in *Bos taurus* cows. Sahiwal cow is one of the established milk breeds of zebu cattle, representing *Bos indicus* [30]. There is very little information regarding luteal dynamics based on LBF in *Bos indicus* dairy cows. Therefore, the objective of this study was to determine if there are differences in luteal size (LS), progesterone (P4), and luteal blood flow (LBF) between pregnant and non-pregnant *Bos indicus* dairy cows during the first three weeks after insemination, and whether these parameters are related to each other.

Materials and Methods

Animal care and management

This study was approved by the Ethics Committee for the Care and Use of Experimental Animals at University of Veterinary and Animal Sciences, Lahore, Pakistan. The study was carried out during the breeding season (March–June 2015) at the Livestock Experimental Station, Jahangirabad, district Khanewal, Punjab, Pakistan. Lactating Sahiwal cows ($n = 13$) of mixed parity (1–3) and body weight 430 ± 18 kg (mean \pm SD), with healthy, regular cycles were used for the study. Body condition score (BCS) was assessed at the start of the study using a 5-point scale: 1 = emaciated to 5 = obese [31]. Cows with a mean BCS of 3.5 ± 0.3 (3.25–3.75) were included in the experiment. The cows were housed in a semi-loose housing system and fed 30–40 kg green fodder (*Trifolium alexandrinum*), 2–3 kg concentrate, and 100 g mineral supplements per day and offered water *ad libitum*.

Study design

Estrus detection was carried out twice daily (0600 h and 1800 h) using a penile-deviated teaser bull to determine the onset of standing heat (day 0). B-mode ultrasonography (My Lab30 Gold VET, Esaote, Genoa, Italy) was performed to confirm the presence of a pre-ovulatory follicle (POF). All cows were artificially inseminated using frozen semen, 12 h after the onset of standing estrus. B-mode ultrasonography was performed repeatedly at 12-h intervals to confirm the absence of POF (ovulation), and this day was referred as day 1 of the estrous cycle. All inseminated cows were retrospectively classified as pregnant (embryonic heartbeat on day 30; $n = 8$) or non-pregnant (inter-estrus interval 17 to 21 days, $n = 5$). In order to compare the LS and LBF after AI, B-mode and color Doppler ultrasonography of the ovaries were performed on days 4, 5, 6, 7 (first week), 8, 10, 12, 14, (second week), and 16, 17, 18, 19, 20, 21 (third week) in pregnant and non-pregnant cows. During the first and third week, sonographic examinations were carried out daily, whereas during the second week, cows were examined on alternate days. Thus, each cow was mapped 14 times, resulting in a total of 182 measurements from all cows.

B-mode and Doppler sonography for luteal dynamics

B-mode ultrasonography of ovaries was performed using a 7.5 MHz linear array transrectal probe. All ultrasound examinations were conducted by the same operator (MH) between 0700 h to 1100

h and took about 25 min for each cow. The corpus luteum (CL) was localized on the ovary and three cross sectional images with maximum areas of the CL were displayed, recorded, and digitized using B-mode sonography. Color Doppler mode was used to assess and ensure the maximum LBF value. Three cross sectional images of the CL were frozen and recorded, avoiding flash artifacts. In order to minimize the variation caused by technical reasons, a standardized preset of the ultrasound machine was used throughout the color Doppler sonographic examinations.

Data analysis and quantification of images

Both B-mode and Doppler mode images of the CL and LBF were recorded in bitmap-format and stored on a hard drive in the ultrasound machine. The stored images were then subjected to offline analysis using computer-assisted image analysis software Image J (National Health Institute, Bethesda, MD, USA). The CL diameter was calculated as described earlier [32]. Each CL was cropped from the captured grey scale image for pixel analysis to measure the cross-sectional area of LS using Image J. The same software was used to quantify the area of color pixels within the CL between pregnant and non-pregnant cows (Fig. 4), which was considered as a semi-quantitative parameter of LBF. In order to minimize the chances of error, mean values of the three images were recorded during each examination.

Blood samples and progesterone assay

Blood samples from the jugular vein were collected after sonographic examinations from each cow. An 18-gauge, 3.8-cm long hypodermic needle in a 10-ml syringe without anticoagulant was used to collect the blood. The serum was separated within 30 min and stored at -20 °C until analysis. Serum concentrations of P4 were measured by using a commercially available double antibody radioimmunoassay kit (ImmunoTech, Prague, Czech Republic) with a 125 I-labelled tracer. The inter-assay and intra-assay coefficients of variation were 6.2% and 3.5%, respectively.

Statistical analysis

Normal distribution of the data was verified using Kolmogorov-Smirnov and Shapiro-Wilk tests. Mean values of LS, P4, and LBF were subjected to analysis of variance (ANOVA) of repeated measurements, considering the variance among the cows during three weeks post AI and using the PROC GLIMMIX procedure to determine the effects of day, group (pregnant and non-pregnant), and the group-time interaction. Least significant difference (LSD) was used as a post-hoc test to determine the difference between the days of estrous cycle. Relationships among LS, concentrations of P4, and LBF of both pregnant and non-pregnant cows during first, second, and third weeks were analyzed using Pearson's correlation coefficient. Correlation coefficients were classified as very good ($r > 0.85$), good ($0.75 < r \leq 0.85$), and moderate ($0.50 < r \leq 0.75$). The data for LS, P4, and LBF were presented as mean \pm standard errors of mean (S.E.M). All data were analyzed using Statistical Analytical System (SAS 9.2 Institute, Cary, NC, USA). Probability level of $P < 0.05$ was considered significant.

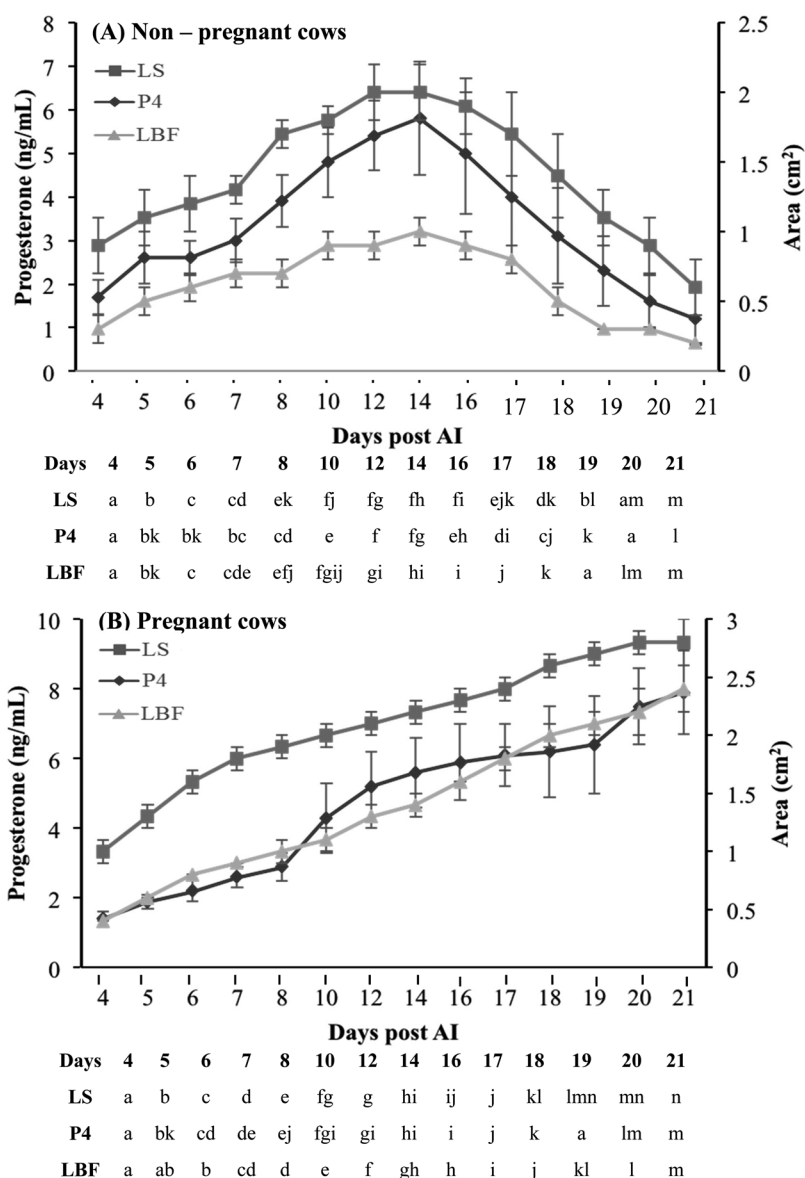


Fig. 1. (A) Represents the absolute changes in luteal size (LS), concentration of progesterone (P4), and luteal blood flow (LBF) of non-pregnant *Bos indicus* dairy cows (n = 5) and (B) signifies the relationships of LS, P4, and LBF in pregnant cows (n = 8). Different letters indicate significant ($P < 0.05$) differences between the respective days.

Results

Cyclic changes in luteal size, progesterone, and luteal blood flow

Non-pregnant cows (n = 5): Changes in LS, P4, and LBF during the first three weeks post AI in non-pregnant cows are presented in Fig. 1A and Fig. 2 (A–C). The mean LS increased ($P < 0.05$) from 0.9 cm² (day 4) to 1.3 cm² (day 7) during the first week, and from 1.7 cm² (day 8) to 2.0 cm² (day 14) in the second week, whereas it declined ($P < 0.05$) from 1.7 cm² (day 17) to 0.6 cm² (day 21) during the third week after AI. The mean LS did not differ ($P > 0.05$) between days 4 (first week) and day 19 (third week), or across days

10, 12, 14, and 16.

The concentrations of P4 rose significantly from 1.7 ng/ml (day 4) to 3.0 ng/ml (day 7) during the first week and increased ($P < 0.05$) further from 3.9 ng/ml (day 8) up to 5.8 ng/ml (day 14) in the second week post AI. In the third week post AI, P4 decreased ($P < 0.05$) from 4.0 ng/ml (day 17) to 1.2 ng/ml (day 21). None of the cows had a P4 level below 1 ng/ml on day 21. The P4 did not differ ($P > 0.05$) between days 4 and 19 and between days 12 and 14.

The LBF increased ($P < 0.05$) from 0.3 cm² (day 4) to 0.7 cm² (day 7), indicating a two-fold rise within 3 days of the first week. In the following days, LBF rose ($P < 0.05$) from 0.7 cm² (day 8) to

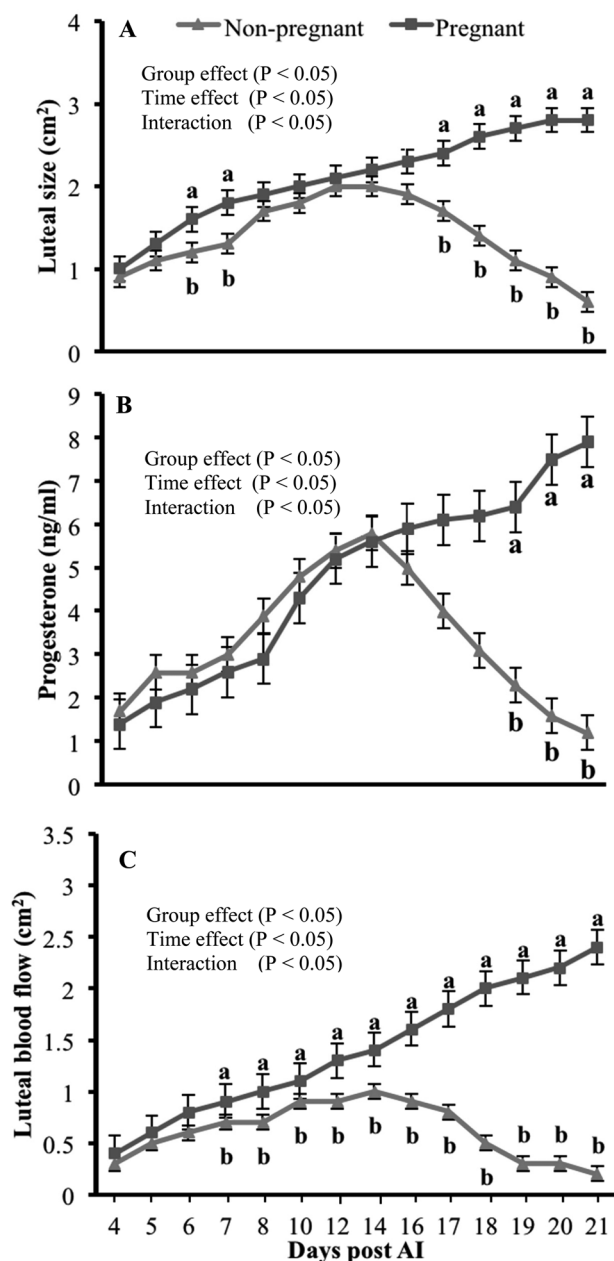


Fig. 2. (A–C) Represents the absolute changes in luteal size (LS), concentrations of progesterone (P4), and luteal blood flow (LBF) in the first three weeks post AI in pregnant and non-pregnant *Bos indicus* dairy cows. Different letters indicate significant ($P < 0.05$) differences between the groups.

1.0 cm² (day 14). During the third week, LBF declined ($P < 0.05$) from 0.8 cm² to 0.2 cm² on days 17 and 21, respectively, as shown in Fig. 4 (A–C). However, the LBF values were similar ($P > 0.05$) on days 4 and 18.

Pregnant Cows (n = 8): The pregnancy rate diagnosed at day 30 post AI using B-mode ultrasonography was 62% (8/13). The changes in LS, P4, and LBF during the three weeks post AI in pregnant

cows are presented in Fig. 1B and in Fig. 2 (A–C). All parameters (LS, P4, and LBF) were affected by time (days; $P < 0.0001$), group (pregnant vs. non-pregnant; $P < 0.0003$), and group-time interactions ($P < 0.001$). The mean LS increased significantly from 1.0 cm² (day 4) to 1.8 cm² (day 7) during the first week, and from 1.9 cm² (day 8) to 2.8 cm² (day 21) during the second and third week post AI. Furthermore, the mean LS was higher ($P < 0.05$) in pregnant cows than in non-pregnant cows on days 6 and 7, and from day 17 onwards (Fig. 2A).

The P4 showed a rise ($P < 0.05$) from 1.4 ng/ml (day 4) to 2.6 ng/ml (day 7) during the first week and increased ($P < 0.05$) further from 2.9 ng/ml (day 8) up to 5.6 ng/ml (day 14) in the second week post AI. Moreover, P4 consistently rose ($P < 0.05$) from 5.9 ng/ml (day 16) to 7.9 ng/ml (day 21) during the third week post AI. Mean concentrations of P4 differed ($P < 0.05$) from day 19 post AI between pregnant and non-pregnant cows (Fig. 2B).

LBF increased continuously during the first three weeks post AI in pregnant cows. The LBF more than doubled ($P < 0.05$) from day 4 (0.4 cm²) to day 7 (0.9 cm²), as well as from day 8 (1.0 cm²) to day 21 (2.4 cm²), as shown in Fig. 4 (D–F). The LBF remained consistently higher ($P < 0.05$) during days 7 through 21 in pregnant as compared to non-pregnant cows (Fig. 2C).

Correlation among relative changes in LS, concentrations of P4, and LBF

Non-pregnant cows (n = 5): Correlations between LS, P4, and LBF in non-pregnant cows are presented in Fig. 3 (A–C). A very good positive correlation ($r = 0.98$; $P < 0.05$) was seen between LS and P4 from days 4 to 20 as well as during the first ($r = 0.98$; $P < 0.05$) and the third week ($r = 0.99$; $P < 0.05$). Likewise, a very good but non-significant correlation was found during the second week ($r = 0.90$; $P > 0.05$) post AI. Very good positive relationships were also noted between P4 and LBF from days 4 to 20 ($r = 0.95$; $P < 0.05$) as well as during the first ($r = 0.93$; $P < 0.05$) and the third week ($r = 0.96$; $P < 0.05$). In the second week after AI, LBF and P4 showed good positive relationships ($r = 0.82$; $P > 0.05$).

A very good correlation was noted between LS and LBF ($r = 0.94$; $P < 0.05$) during days 4 through 20 after AI. Moreover, we observed a good correlation in the first week ($r = 0.84$; $P > 0.05$) and a very good correlation in the third week ($r = 0.93$; $P < 0.05$) after AI. However, only a moderate, non-significant correlation was noted during the second week ($r = 0.52$; $P > 0.05$) after AI.

Pregnant cows (n = 8): Correlations between LS, P4, and LBF in pregnant cows are presented in Fig. 3 (D–F). A very good positive correlation ($r = 0.95$; $P < 0.05$) was observed between LS and P4 from days 4 to 21. Similarly, high correlations were found during the first ($r = 0.98$; $P < 0.05$) and the second ($r = 0.92$; $P < 0.05$) week. A moderate non-significant correlation ($r = 0.74$; $P > 0.05$) was noted during the third week after AI. A very good positive correlation ($r = 0.96$; $P < 0.05$) was observed between P4 and LBF from days 4 to 21 as well as during the first ($r = 0.98$; $P < 0.05$), second ($r = 0.96$; $P < 0.05$), and third ($r = 0.91$; $P < 0.05$) week after AI in pregnant cows. Very good positive correlations were also observed between LS and LBF from days 4 to 21 after AI ($r = 0.97$; $P < 0.05$) and during the first ($r = 0.99$; $P < 0.05$), second ($r = 0.96$; $P < 0.05$), and third ($r = 0.93$; $P < 0.05$) week after AI.

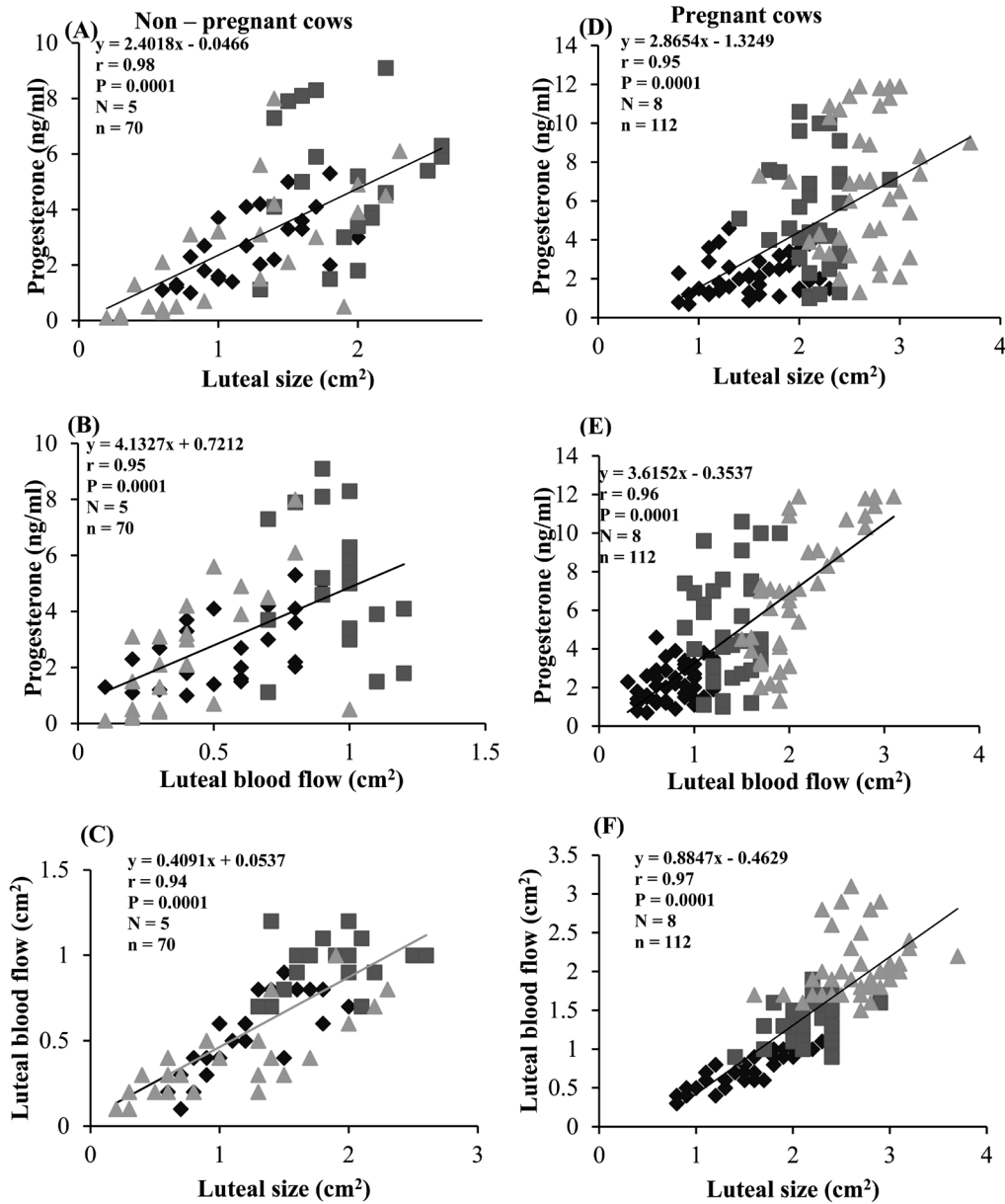


Fig. 3. (A–C) Correlations among luteal size (LS), concentrations of plasma progesterone (P4), and luteal blood flow (LBF) during the first (◆), second (■), and third week (▲) after AI in non-pregnant ($n = 5$) *Bos indicus* dairy cows. (D–F) Correlations among LS, plasma P4, and LBF during the first (◆), second (■), and third week (▲) after AI in pregnant ($n = 8$) *Bos indicus* dairy cows.

Discussion

To the best of our knowledge, this study is the first to report the relationships among LS, P4, and LBF in pregnant and non-pregnant *Bos indicus* dairy cows. The salient finding of this study is that compared with LS or P4, LBF seems a better indicator of differences in luteal function between pregnant and non-pregnant *Bos indicus* cows. This is because LBF consistently began to differ between pregnant and non-pregnant cows on day 7, whereas LS or P4 values diverged at a much later stage, i.e., on days 17 and 19, respectively.

These findings are supported by recent studies in which LBF was significantly higher at day 7 in pregnant Holstein cows [33], and at day 9 in Saanen goats [34]. The most plausible reason for the enhanced LBF could be an increased demand of nutrients and substrates of P4 by the embryo and CL, respectively, during the elongation phase [33]. Recent evidence indicated an increased CL function during early pregnancy in temporal association with an increase in blood flow in the ipsilateral uterine artery of dairy heifers [35]. On the other hand, LBF, during the late luteal phase, was higher at days 15 or 18, although not consistently, in pregnant vs. non-pregnant

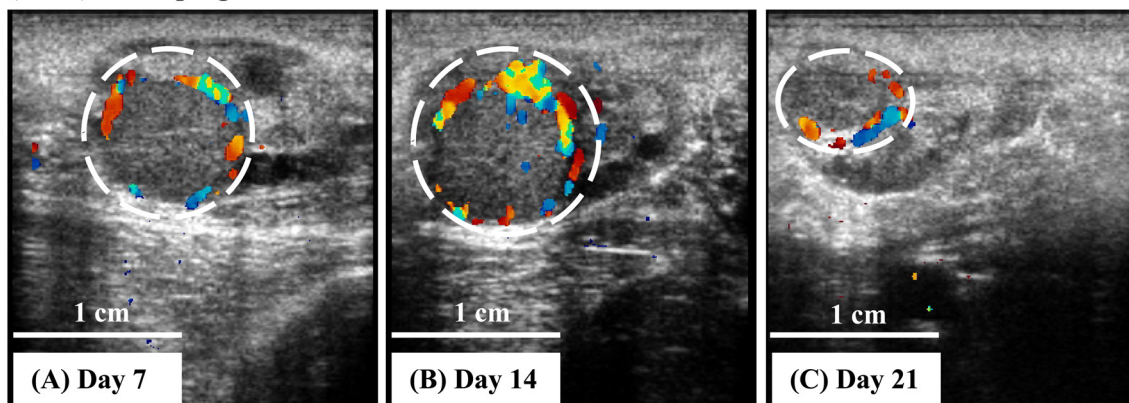
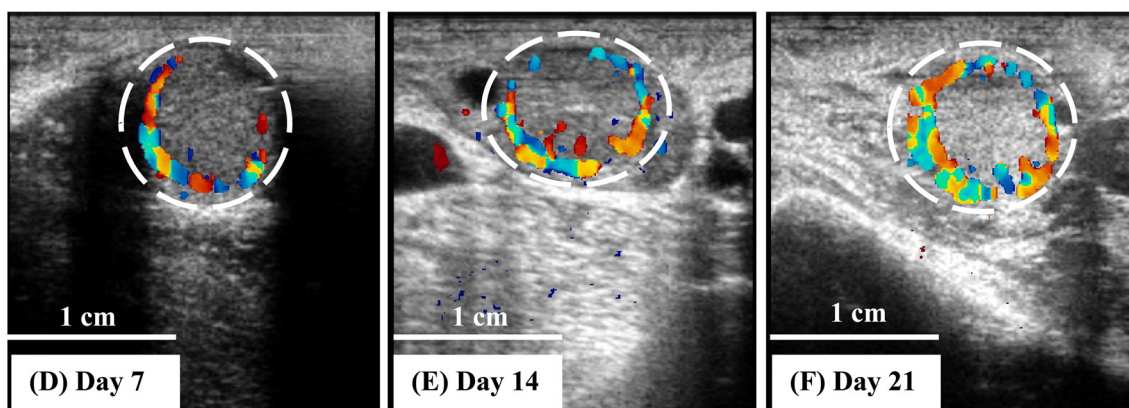
(A - C) Non – pregnant cows**(D - F) Pregnant cows**

Fig. 4. (A) Representative colour Doppler sonograms of CL and LBF during the first (day 7), (B) second (day 16), and (C) third week (day 21) after AI in non-pregnant *Bos indicus* dairy cows. (D) Characteristic colour Doppler sonographic images of CL and LBF during the first (day 7) and (E) second week (day 14), which did not regress. (F) The maximum vascularization on third week (day 21) after AI in pregnant *Bos indicus* dairy cows. The white line circles indicate the region of interest (ROI) used to calculate the cross-sectional area of the CL. Within the ROI, the coloured pixels were quantified using image J software (National Institute of Health, Bethesda, MD, USA) to assess the maximal LBF.

and non-bred dairy cows [24]. The variability in outcomes could be attributed to species differences. This warrants further studies in different species, in order to establish the relationships among LBF, embryo, and P4 during first three weeks post AI.

The present study indicated that LBF declined dramatically on day 17 after AI in non-pregnant cows. This finding is in accordance with the previous reports where LBF was shown to decrease between days 17 and 21 in non-pregnant crossbred beef cows [23], German Holstein cows [19], Holstein Friesian cows [12], and beef heifers [27]. Previously, it was reported that there is an initial transient increase [17] followed by a decline in LBF within 24 h due to enhanced secretion of PGF2 α [36], which is an established vasoconstrictor [37]. Moreover, it has been reported that decrease in LBF after luteolysis renders the synthesis of P4 from the luteal cells [19]. Thus, a visual evaluation of LBF in third week represents a quick, reliable, and consistent diagnostic test that would enable the detection of non-pregnant cows [23]. It would be interesting to see if administration of PGF2 α inhibitors enhances the LBF during the third week after

AI in *Bos indicus* cows.

In the present study, LS showed a significant increase during the first week post AI but remained similar until luteolysis in pregnant and non-pregnant cows. This revealed that CL grows rapidly in pregnant and slowly in non-pregnant cows during the first week post AI. This rapid growth is associated with intensive angiogenesis in early CL due to the abundance of rough endoplasmic reticulum in the cytoplasm of endothelial cells with a loose arrangement in the network of capillaries [38]. Both LBF as well as LS served as an early indicator of CL function compared with P4, which started to differ only from day 19 post AI between pregnant and non-pregnant cows. This might be because P4 can only be measured in the peripheral blood and depends not only on the synthesis of P4 from CL, but also on the metabolism in the liver [39].

The present study validated the highly correlated inter-relationships among LS, P4, and LBF during the first three weeks after AI in both pregnant and non-pregnant *Bos indicus* dairy cows. These observed high correlations are not surprising and were expected,

as all three parameters are dependent on one structure, i.e., CL. The high association between LBF and P4 in this study, is in close agreement with the earlier results, where correlations were strong between pregnant and non-pregnant Holstein cows [19], dairy heifers [27], and Saanen goats [34]. Traditionally, these variables showed moderate correlation when measured through rectal palpation [40], good correlation when determined via B-mode ultrasonography [41], and very good correlation when measured using CDU [42]. With the advent of CDU, it has now been established that LBF represents an efficient indicator of luteal function. The availability of this information may serve as the basis for the development of disruptive technology in reproduction of dairy cows.

From a practical point of view, early diagnosis of pregnancy is extremely important for proficient reproductive management. The evaluation of LBF on day 17 after AI might be useful for early estrus detection and re-insemination of non-pregnant cows [12]. Previously, it has been reported that LBF on day 21 could be used for resynchronization 9 to 14 days early, compared with the conventional management based on pregnancy diagnosis at days 30 to 35 [13]. In this case, no estrus detection would be required, and AI would occur at the same time as the expected natural return to estrus. Thus, strategies for rapid resynchronization after a diagnosis of non-pregnancy using the LBF on day 17 must be developed and tested on large-scale in dairy herds.

In conclusion, the present study provided new data about the relationships among LS, P4, and LBF, and indicated that LBF is a more sensitive parameter than LS or P4 to detect the differences in luteal function during the first three weeks after AI between pregnant and non-pregnant *Bos indicus* dairy cows. Furthermore, this approach could be effectively used to decrease the re-insemination interval, number of open day, and calving interval, for the optimization of reproductive management in dairy cows.

Acknowledgments

The authors thank Dr Manzoor Ahmad Mangat, Farm Superintendent, Livestock Experimental Station (LES), Jahangirabad, for allowing the use of their cows for this study. The authors are also thankful to Dr Muhammad Shahzad, Scientific Officer, Nuclear Institute for Agriculture and Biology (NIAB) for performing the progesterone assays. This research was supported by a grant from Agricultural Linkages Program (ALP), Pakistan Agricultural Research Council (PARC), Islamabad, Pakistan.

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