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Doppler sonographic vascular and morphometric profiling of folliculo-luteal dynamics in subclinical endometritic indigenous cattle

Uttam Kumar Sahu[1], Brijesh Kumar[1]*, Meraj Haider Khan[1]*, Chinmay Warghat[1], Mayank Singh Baghel[1], Vishnu Vadera[1], Amit Kumar[1,2], Laxmi Sahu[3], Mahesh Kumar[4], Nitish Singh Kharayat[5], Sanjay Kumar Singh[1]

[1] Division of Animal Reproduction, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.), India; [2] ICAR-Indian Institute of Agricultural Biotechnology, Ranchi, (J.H.), India; [3] Livestock Production and Management, College of Veterinary Science and Animal Husbandry, Anjora, Durg, Chhattisgarh, India; [4] Division of Surgery, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.), India; [5] Temperate Animal Husbandry Division, ICAR- Indian Veterinary Research Institute, Muksteshwar Campus, Nainital, Uttarakhand, India.

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Abstract

Subclinical endometritis (SCE), driven uterine dysbiosis, impairs fertility by reduced conception, embryonic survival and overall reproductive efficiency. The ovarian sequelae of SCE in *Bos indicus* remain poorly defined. Tharparkar cows (n = 20) were enrolled and monitored across one estrous cycle using transrectal B-mode and Doppler ultrasonography. Animals were classified as SCE (n = 10) or healthy (n = 10) based on endometrial polymorphonuclear neutrophil proportions (> 5.00%), a threshold justified by breed-specific postpartum uterine adaptation in *B. indicus*. Polymorphonuclear neutrophils were significantly higher in the SCE group (6.80 ± 0.24% *vs.* 3.30 ± 0.21%). SCE cows exhibited reduced medium and total follicle numbers and slower dominant follicle growth (0.89 ± 0.05 *vs.* 1.02 ± 0.03 mm *per* day). Mean corpus luteum (CL) pixel area was consistently lower in the SCE group throughout the luteal phase. The CL vascularity indices derived from color Doppler mode (red = toward, blue = away from transducer) were reduced (*e.g.*, day three perfusion: 24.94 ± 2.75% *vs.* 17.71 ± 2.34%). Serum progesterone was lower in SCE cows and strongly correlated with CL size (Pearson's correlation coefficient [r] = 0.89; 95.00% confidence interval, [CI] = 0.84 - 0.92) and perfusion (r = 0.50, 95.00% CI 0.35 - 0.62). In conclusion, SCE in *B. indicus* compromised follicular dynamics, early CL development, vascularization and steroidogenesis. Pixel-derived Doppler indices provided robust quantitative markers for early detection and targeted management of SCE.

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Introduction

Reproductive efficiency is fundamental to dairy herd profitability and depends on uterine health, timely estrus detection, conception success and optimal calving intervals. Despite India leading milk production volume, the national average yield (4.87 kg *per* day) remains lower than the global average (7.20 kg *per* day), largely due to reproductive inefficiency and infertility in farm animals.[1] Postpartum uterine infections contribute to significant economic losses estimated at approximately $180 million USD annually and impair overall herd productivity.[2] For cows to achieve peak lactation yield and maintain reproductive efficiency, they must overcome three major postpartum challenges: Uterine involution, resumption of ovarian cyclicity and negative energy balance.[3] Postpartum uterine diseases include metritis, clinical endometritis, and subclinical endometritis (SCE), with the latter being the most insidious due to the lack of clinical signs. Globally, the incidence of SCE ranges from 10.00 - 30.00% in *Bos taurus* dairy cows and 5.00 - 20.00% in *B. indicus,* depending on postpartum period, diagnostic methods and herd management practices.[4-6] The SCE disrupts ovarian function via endotoxin transfer through the utero-ovarian vasculature, a phenomenon documented in *B. taurus.*[7] Accumulation of lipopolysaccharides (LPS) and inflammatory mediators like tumor necrosis factor-alpha (TNF-α) in the follicular fluid impairs granulosa cell

***Correspondences:**
Brijesh Kumar. BVSc, AH, MVSc, PhD
Division of Animal Reproduction, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.), India
E-mail: brijesh.kumar3@icar.org.in
Meraj Haider Khan. BVSc, AH, MVSc, PhD
Division of Animal Reproduction, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.), India
E-mail: haider.meraj@icar.org.in

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aromatization of androstenedione, reduces estradiol synthesis, increases follicular atresia, blunts follicle-stimulating hormone responsiveness and inhibits aromatization without affecting androgen production.[8] Uterine infection has also been associated with compromised dominant follicle growth, prolonged luteal phases and reduced estradiol and progesterone secretion.[8] While wave emergence remains unaffected, dominant follicles in affected cows grow more slowly and secrete less estradiol.[9] Experimental LPS infusion during diestrus reduced luteal area from 5.20 to 3.80 cm^2 and decreased blood flow by 34.00%, corroborated by *in vitro* evidence of LPS-mediated impairment of luteal angiogenesis and endothelial integrity.[10-12] However, there remains a paucity of Doppler ultrasonography-based profiling of follicular-luteal dynamics in *B. indicus* Tharparkar cattle, a hardy dual-purpose indigenous breed valued for exceptional heat tolerance (temperature-humidity index threshold ≈ 80 *vs*. 72 in *B. taurus*), drought resilience, sustained milk yield under tropical stress.[13,14] Additionally, study reports earlier uterine involution and lower baseline neutrophilic infiltration postpartum in *B. indicus* compared to *B. taurus.*[15] These, physiological and genetic superiority, make Tharparkar cattle crucial for improving dairy productivity and climate adaptability in tropical and arid regions. Breed-specific differences in uterine–ovarian interactions, vascular dynamics and endocrine responses to uterine infections are poorly under-stood. This study provided, to our knowledge, the first Doppler ultrasonography-based characterization of SCE-induced alterations in ovarian folliculo–luteal dynamics in indigenous Tharparkar cows, addressing a critical knowledge gap in *B. indicus* reproductive physiology.

Materials and Methods

Location and climatic parameters. The experiment was conducted at the Cattle and Buffalo Farm, ICAR–IVRI, Izatnagar, India (28° N, 79° E; 564 m above mean sea level). The climate of the region is humid and tropical, with a mean summer temperature of 30.70 ˚C (range 15.10 - 40.90 ˚C) and mean relative humidity of 53.20%.

Animals and feed management. The study involved twenty (n = 20) Tharparkar cows (parity 1 - 3; age 4.80 ± 0.80 years; body weight 388 ± 9.00 kg; milk yield 5.23 ± 1.40 kg *per* day) selected for Doppler ultrasonography. Cows received 1.50 - 2.50 kg *per* day concentrate (20.00% Digestible Crude Protein, 70.00% Total Digestible Nutrients), *ad libitum* wheat straw and green fodder twice daily with free access to water. A mineral–vitamin mixture (Indian Council of Agricultural Research standard) was provided daily. Animals were housed under semi-intensive management with access to open paddocks.

Experimental design and estrus monitoring. All animals underwent clinical screening to exclude systemic or inflammatory conditions. Reproductive tract evaluation included visual vulvar inspection and vaginal palpation, and clear mucus indicated absence of clinical endometritis. Animal found positive for uterine infections were excluded from the study. Only clinically normal cows with regular estrous cycles, no history of uterine infection, dystocia or retained placenta and no abnormal discharge were included. Transrectal palpation and B-mode ultrasonography confirmed uterine integrity, with no intrauterine fluid or abnormalities. No hormonal synchronization of estrous cycles was performed. Each cow was enrolled on a random day of its cycle and daily ultrasonographic monitoring was initiated to track the growth of the dominant follicle. Once natural behavioral estrus was detected, confirmed by the presence of a preovulatory follicle and clear mucous discharge, the animal study parameters were recorded and the monitoring continued throughout one estrous cycle. This approach allowed physiological characterization of follicular–luteal dynamics under natural estrous conditions, without exogenous hormonal intervention. Subclinical endometritis was diagnosed by endometrial cytology using the cytobrush technique.[4,15] Smears were stained with modified Wright's Giemsa, and 100 cells *per* slide were counted. Based on established cytological criteria, SCE was defined as the presence of > 5.00% polymorphonuclear neutrophil (PMN)s at ≥ 47 days postpartum,[4] additionally, based on breed-specific postpartum adaptation in *B. indicus* supported by an author.[15] Ten cows with SCE formed the treatment group and ten healthy cows served as controls. Post-cytobrush welfare monitoring was performed for 24 hr using a standardized 5-point pain score and observation for abnormal discharge as *per* ARRIVE 2.0 guidelines.[16] No adverse reactions were noted. All experimental procedures were approved by the Institute Animal Ethics Committee of ICAR-IVRI, Izatnagar, U.P. India (26-3/2020-21/JD(R)/IAEC).

Doppler ultrasonography monitoring of follicular and luteal dynamics. Cows were monitored daily by rectal palpation and transrectal B-mode and color Doppler ultrasonography using an Exago ultrasound system equipped with a 7.5 MHz linear transrectal transducer (ECM, Angoulême, France) throughout one estrous cycle. Observer reliability was assessed weekly, intra- and inter-observer intraclass correlation coefficients were 0.92 and 0.88, respectively. Follicular wave emergence was identified when ≥ 3.00 mm follicles appeared as a cohort, with at least one reaching ≥ 5.00 mm within 48 hr.[17] Follicles were classified as small (< 5.00 mm), medium (5.00 - 9.00 mm) or dominant (≥ 9.00 mm). The day of estrus behavior was designated as Day –one and ovulation (Day 0) was confirmed by disappearance of the pre-ovulatory follicle.

The corpus luteum (CL) diameter was measured daily, categorizing the luteal phases into growth (days 3 - 8),

static (days 9 - 14) and regression (days 15 - 18).[18] Follicle and CL diameters were calculated as the mean of two perpendicular measurements using inbuilt callipers. Total luteal tissue area (mm^2) was calculated excluding the cavity area.[19]

$$Total\ luteal\ tissue = \frac{Length\ (mm)}{2}, \frac{Width\ (mm)}{2} \times \pi$$

Follicle growth rate (FGR; mm per day) and regression rate (FRR; mm *per* day) of dominant follicle were determined as described prevoiusly:[20]

$$FGR = \frac{Maximum\ diameter\ (mm) - Minimum\ diameter\ (mm)}{Number\ of\ days\ of\ growth}$$

$$FRR = \frac{Maximum\ diameter\ (mm) - Minimum\ diameter\ (mm)}{Number\ of\ days\ of\ regression}$$

Estimation of luteal blood perfusion and total luteal tissue. Luteal vascularization was evaluated using color Doppler mode (red = flow toward, blue = flow away from transducer) ensuring consistent delineation of perfusion patterns. Vascularized areas were delineated using the magnetic tool in Adobe Photoshop CS5 (version 12.0; Adobe Systems Inc., San Jose, USA), and pixel counts were obtained.[21] Validation against ImageJ was performed in 10 randomly selected images, yielding > 95.00% agreement, confirming reproducibility. Luteal blood flow (%) was calculated as the ratio of colored area to total CL area.[22] Cavity areas were excluded deliberately to prevent overestimation of functional luteal tissue as previously reported in bovine luteal morphometry studies.[22,23]

Blood sampling and progesterone estimation. Following each Doppler exam, 4.00 mL blood was collected by jugular venipuncture into clot activator tubes, allowed to clot for 30 min, and centrifuged at 3,000 rpm for 10 min. Serum was stored at – 20.00 ˚C. Progesterone was quantified using a DRG Progesterone ELISA kit (EIA-1561; DRG Instruments GmbH, Marburg, Germany). Assay sensitivity was 0.045 ng mL^{-1}; intra- and inter-assay coefficients of variation were 6.40 and 6.60%, respectively. All samples were analyzed in duplicate.

Statistical analysis. Data were analyzed using GraphPad Prism (version 10.6; GraphPad Software Inc., San Diego, USA). Data normality was checked by Shapiro-Wilk and Kolmogorov–Smirnov tests. The PMN counts, follicle numbers, dominant follicle and CL dynamics, pre-ovulatory follicle and CL size, luteal perfusion, luteal phase length, estrous cycle length and progesterone (P4) levels were compared using independent t-tests. Time-series analysis using repeated measures ANOVA was also performed for luteal parameters and serum P4 values, and comparison was done with post hoc Tukey's tests. Also, power analysis was performed using G*Power (version 3.1; Heinrich Heine University Düsseldorf, Düsseldorf, Germany), which indicated that n = 10 *per* group provided 80.00% power to detect a 20.00% difference in luteal perfusion indices at α = 0.05. Additionally, Pearson's correlations between P4 and CL size or luteal perfusion were calculated. Data were expressed as mean ± SEM and $p < 0.05$ was considered statistically significant.

Results

Polymorphonuclear neutrophil counts in endometrial cytology screening. Ten cows with a mean PMN count of 6.80 ± 0.24% tested positive for SCE, while ten healthy animals with a mean PMN count of 3.30 ± 0.21% were included in the control group. The PMN count in the SCE group was significantly higher ($p < 0.0001$) than in the healthy group (Table 1).

Morphometric follicular attributes. The SCE did not significantly affect ($p > 0.05$) the number of small or large follicles. However, medium follicle counts and total follicular population were significantly lower in cows with SCE compared to healthy controls ($p < 0.05$; Table 1). Preovulatory follicle size did not differ significantly between groups ($p > 0.05$). Cows with SCE exhibited slower dominant follicle growth rates compared to healthy cows ($p < 0.05$), while regression rates were numerically higher but not statistically significant ($p > 0.05$). For these non-significant differences, effect sizes (Cohen's d) were moderate (0.48 - 0.55), indicating potential biological relevance despite lack of statistical significance.

Table 1. Polymorphonuclear neutrophil counts, follicular and luteal attributes in healthy and subclinical endometritis (SCE)-affected Tharparkar cows (mean ± SEM).

Parameters	Healthy (n = 10)	SCE (n = 10)	*p*-value
Polymorphonuclear neutrophil count (%)	3.30 ± 0.21	6.80 ± 0.24	< 0.05
Number of small follicles (< 5.00 mm) *per* animal (both ovaries)	15.36 ± 0.84	13.68 ± 0.62	> 0.05
Number of medium follicles (5.00 - 9.00 mm) *per* animal (both ovaries)	1.81 ± 0.33	1.00 ± 0.18	< 0.05
Number of large follicles (≥ 9.00 mm) *per* animal (both ovaries)	0.68 ± 0.10	0.54 ± 0.10	> 0.05
Total number of follicles *per* animal (both ovaries)	17.86 ± 0.81	15.23 ± 0.71	< 0.05
Dominant follicle growth rate (mm *per* day)	1.02 ± 0.03	0.89 ± 0.05	< 0.05
Dominant follicle regression rate (mm *per* day)	0.84 ± 0.05	0.94 ± 0.06	> 0.05
Preovulatory follicle size (mm)	13.14 ± 0.31	12.68 ± 0.28	> 0.05
Corpus luteum growth rate (mm *per* day)	1.02 ± 0.08	0.93 ± 0.05	> 0.05
Corpus luteum regression rate (mm *per* day)	1.12 ± 0.07	1.06 ± 0.05	> 0.05
Luteal phase duration (days)	16.10 ± 0.23	17.30 ± 0.21	< 0.05
Estrous cycle duration (days)	20.50 ± 0.30	21.60 ± 0.37	< 0.05

Vascular and morphometric attributes of the CL. Cows without SCE consistently exhibited larger CL diameters than SCE-affected cows from day three through day 18, except on day nine ($p > 0.05$). Luteal tissue area followed the same pattern with significantly smaller areas in SCE cows at all stages except day nine (Figs. 1A and 1B). Time-series analysis using repeated measures ANOVA showed a significant day effect ($p < 0.01$) for CL perfusion, with post hoc Tukey's tests identifying day three as significantly lower than days six and 12. The CL pixel area was consistently reduced in SCE cows across the luteal phase (Fig. 1C). Similarly, luteal vascularization area in pixel was significantly lower on days three, six, 12 and 15 ($p < 0.05$), and percentage luteal blood perfusion differences were most pronounced on day three ($p < 0.05$; Figs. 1D, 2A, and 3). The mean CL growth rate was numerically lower in SCE cows than in healthy cows, however, the difference was not significant ($p > 0.05$; Cohen's d = 0.41; Table 1). Regression rates were also slower in SCE-affected cows (1.06 ± 0.05 mm *per* day) than in healthy cows without reaching significance ($p > 0.05$; Cohen's d = 0.36).

Progesterone dynamics. Serum P4 concentrations were significantly lower in SCE-affected cows ($p < 0.05$) at multiple time points: day zero (ovulation), early luteal phase (day three, day six), mid-luteal phase (day 12) and late luteal phase (day 15; Fig. 2B). Strong positive correlations were observed between P4 concentration and CL size (Pearson's correlation coefficient [r] = 0.89; 95.00% confidence interval, [CI] = 0.84 - 0.92, $p < 0.0001$) and between P4 and luteal perfusion (r = 0.50, 95.00% CI 0.35 - 0.62, $p < 0.001$), confirming the physiological coupling of structure, perfusion and function (Figs. 2C and 2D).[24]

Duration of luteal phase and estrous cycle. The luteal phase was significantly prolonged in cows with SCE compared to healthy cows ($p < 0.05$), (Table 1). Similarly, estrous cycle length was extended in SCE-affected cows ($p < 0.05$) indicating delayed luteolysis and cycle progression.

Discussion

This study provided the first Doppler ultrasonography-based evidence of SCE-associated alterations in follicular and luteal dynamics in *B. indicus* Tharparkar cattle. A significant reduction in medium-sized follicles and total follicular counts was observed in SCE-affected cows. This pattern aligned with established inflammatory mechanisms in dairy cattle, where uterine-derived mediators such as TNF-α and LPS impair gonadotropin-releasing hormone/luteinizing hormone secretion, disrupting estradiol synthesis required for follicular growth.[25] Although small follicles showed a non-significant reduction, the potential impact on primordial follicle pools warrants further mechanistic studies.

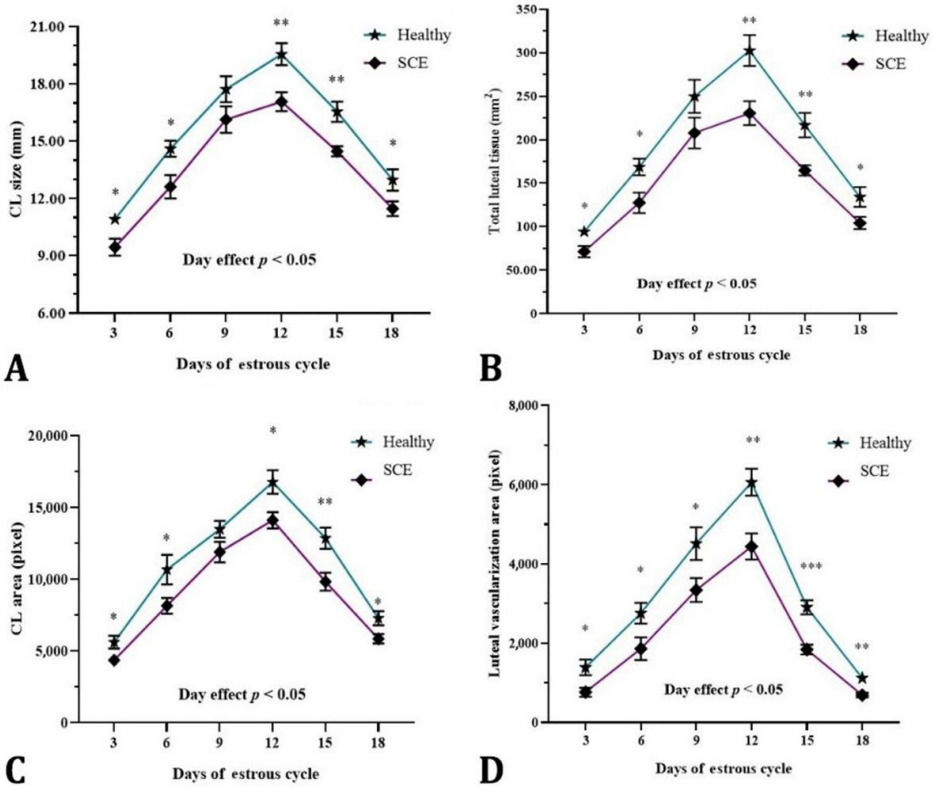


Fig. 1. Vascular and morphometric attributes of Corpus luteum (CL) in Tharparkar cows **; A)** CL size (mm) and; **B)** Total luteal tissue (mm²), measured via ultrasonography; **C)** CL area (pixel), and; **d)** luteal vascularization area (pixel) quantified in pixels using image analysis software. Value with asterisk (*) were statistically significant ($p < 0.05$).

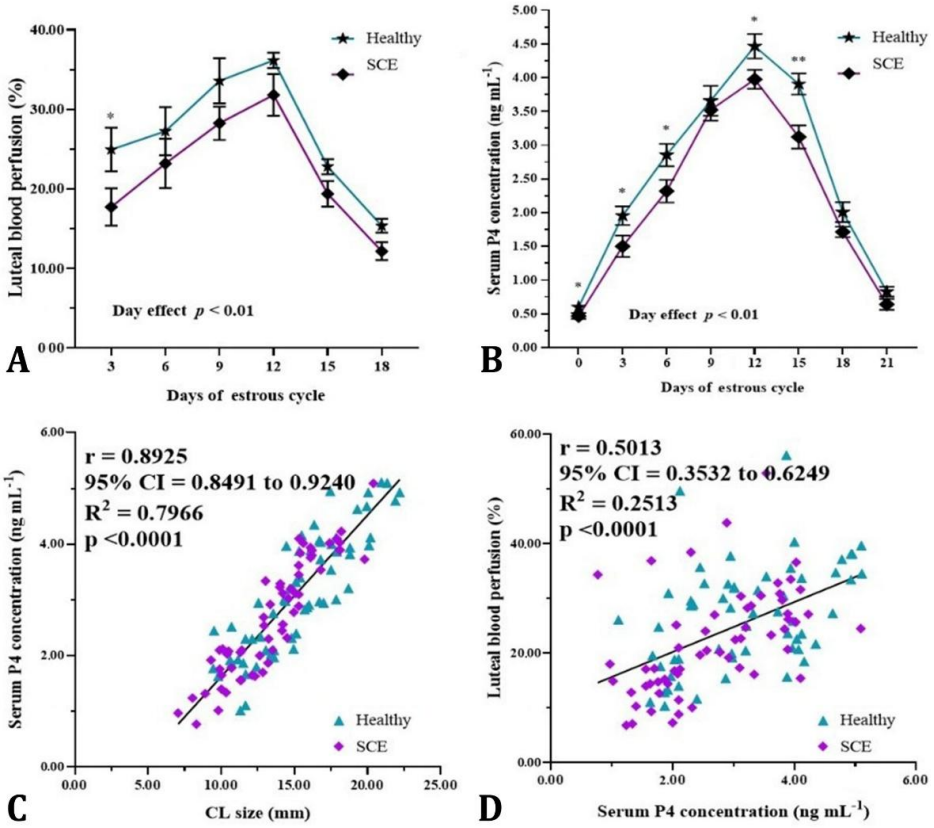


Fig. 2. Vascular and morphometric attributes of Corpus luteum (CL) and progesterone (P4) dynamics and their correlation in Tharparkar cows; **A)** Luteal blood perfusion (%); **B)** Serum P4 (ng mL^{-1}); **C)** Correlation between serum P4 and CL size; **D)** Correlation between luteal blood perfusion and serum P4 level. Value with asterisk (*) were statistically significant ($p < 0.05$).

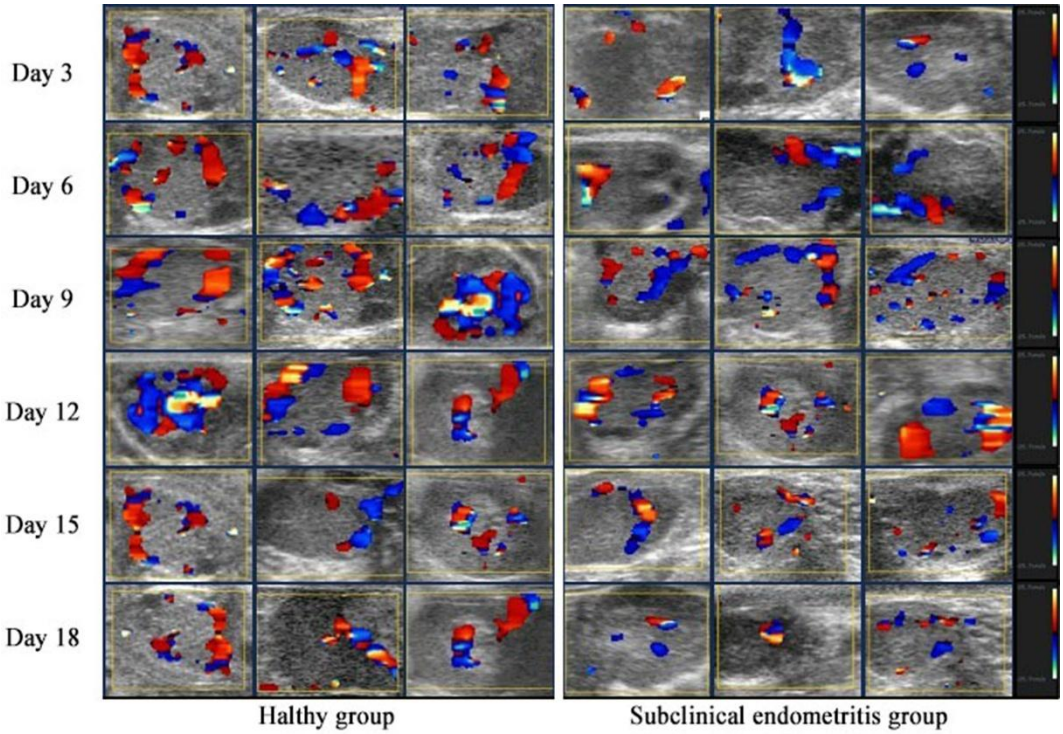


Fig. 3. Doppler ultrasonography based luteal blood perfusion assessment on a perfusion scale bar of – 25.70 to + 25.70 cm *per* sec between two groups during day 3 to 18.

Notably, emergence of follicular waves were preserved, consistent with earlier reports,[9,26] suggesting that cytokine-mediated disruption in *B. indicus* might specifically affect medium follicle development. Dominant follicle size and regression rates did not differ significantly between groups, although mean values indicated smaller size and higher regression rates in SCE cows. Growth rates were significantly reduced, consistent with earlier findings linking genital tract infections to impaired folliculogenesis through LPS- and TNF-α–mediated granulosa cell dysfunction, leading to reduced estradiol secretion and impaired follicle growth.[25,27] Uterine inflammation may also alter the composition of follicular fluid, elevating LPS and TNF-α concentrations, potentially compromising oocyte quality and early embryonic development areas requiring further investigation in *B. indicus*. The SCE also had a marked impact on CL structure and vascularity. Cows with SCE exhibited smaller CL size and reduced luteal tissue area throughout the cycle, reflecting structural suppression associated with inflammatory mediators. The TNF-α and endotoxins released during uterine infection have been shown to directly impair luteal cell function,[9,28] reduce luteal vascularization,[12] and alter Prostaglandin F $(PGF)_2\alpha$ secretion.[29] Collectively, these mechanisms explain the smaller CL size and reduced luteal tissue area observed in SCE-affected cows, consistent with previous reports of uterine infection-associated luteal suppression.[11,12] Luteal blood perfusion was significantly reduced in SCE cows, especially during the early luteal phase. This mirrored earlier work demonstrating a 34.00% reduction in perfusion following endotoxin exposure.[12] *In vitro* studies have confirmed that endotoxin-induced inflammation disrupts luteal angiogenesis and endothelial integrity, downregulating vascular endothelial growth factor, a key angiogenic factor.[10,11,23,30] Color Doppler ultrasonography provided a functional correlation of these changes, as reduced perfusion was associated with lower progesterone levels, consistent with previous findings linking luteal blood flow to luteal function and fertility.[23,31] Uterine infections disrupt CL dynamics through inflammatory and endocrine mechanisms, reducing CL size, growth and P4 secretion postpartum.[9,12,32] In our study, SCE cows showed smaller CL size and lower progesterone concentrations, consistent with previous reports.[12,25,33] This may stem from inflammatory mediators (endotoxins, chemokines) acting directly on luteal cells or indirectly via increased endometrial prostaglandin synthesis, upregulating genes such as Caspase-3, Toll-like receptor 4, TNF-α, PGF synthase, prostaglandin E (PGE) synthase, and vascular endothelial growth factor, while suppressing steroidogenic acute regulatory gene and 3-beta-hydroxysteroid dehydrogenase.[23,30] The luteal phase and estrous cycle were significantly prolonged in SCE affected cows, supporting earlier reports linking uterine pathology to extended luteal function.[12,34] This may be due to impaired luteolysis resulting from inadequate $PGF_2\alpha$ secretion.[35] In ruminants, $PGF_2\alpha$ drives luteolysis, while PGE_2 supports luteal maintenance.[36] Endotoxin exposure shifts the PGE_2:$PGF_2\alpha$ ratio toward luteal maintenance,[35,37] contributing to delayed luteolysis, reduced progesterone synthesis and prolonged estrous cycles in infected cows.[9,20] The present findings paralleled those reported in *B. taurus*,[20,38] yet tropical factors such as heat stress may modulate inflammatory and endocrine responses in *B. indicus*.[13] Although Tharparkar cattle possess inherent heat tolerance, environmental stressors can still alter vascular and hormonal dynamics, warranting careful consideration in future research. To unravel the mechanistic basis of SCE-associated ovarian alterations in indigenous breeds, future studies should incorporate integrative multi-omics platforms, including RNA-seq and proteomics, to dissect cytokine signaling cascades and steroidogenic gene networks. The study was limited to the evaluation of a single estrous cycle without assessment of fertility parameters such as conception rates or embryonic development, and molecular profiling was not conducted. Addressing these limitations will be essential to strengthen causal inferences linking SCE, uterine–ovarian vascular changes and reproductive outcomes.

In conclusion, Doppler ultrasonography demonstrated that SCE in *B. indicus* cows disrupted follicular–luteal dynamics, reduced CL pixel area, luteal vascularization area (pixel) and perfusion percentage. These Doppler-derived parameters serve as sensitive and quantitative indicators of ovarian dysfunction associated with SCE. Further exploration of prostaglandin profiles and inflammatory pathways will be crucial to elucidate the underlying mechanisms more comprehensively.

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Conflict of interest

The authors declare that there is no conflict of interest in publishing the article.

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