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Quantitative Analysis of Small and Large Luteal Cells During Different Stages of Corpus Luteum Development in Holstein Cattle

Bui Van Dung*, Nguyen Thi Thanh Thuy, Nguyen Thi Ngoc Anh, and Nguyen Hoai Nam

Department of Animal Surgery and Theriogenology, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Trau Quy Town, Gia Lam District, Ha Noi, Viet Nam

*Corresponding author's Email: bvdung@vnua.edu.vn

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ABSTRACT

The bovine corpus luteum (CL) undergoes dynamic structural and functional changes throughout its lifespan, driven by distinct populations of steroidogenic cells, including small luteal cells (SLCs) and large luteal cells (LLCs). Although these cell types have been morphologically characterized, quantitative evaluations of their numerical abundance and relative distribution across different developmental phases of the CL in cows remain limited. This study sought to quantify the populations of small and large luteal cells across the early, mid, and late stages of corpus luteum development in Holstein cows. Histomorphometric analysis of hematoxylin and eosin–stained ovarian sections was performed to provide insights into their temporal roles in luteal function. Ovarian samples were obtained from 30 Holstein cows (30 pairs), immediately after slaughter, and categorized into early, mid, or late luteal phases according to ovarian morphology and the appearance of corpora lutea. A total of 30 CL samples (10 per stage) were selected for evaluation. Each CL was fixed, paraffin-embedded, and sectioned longitudinally (5 µm thick) for histological examination. Small (<20 µm) and large (> 35 µm) luteal cells were quantified in standardized microscopic fields at × 40 magnification using ImageJ software. The results revealed that SLC counts were highest during the early luteal phase (72.53 ± 7.83) and significantly exceeded LLC counts (30.70 ± 3.78). In the mid-luteal phase, SLCs decreased (50.70 ± 2.82) while LLCs increased (44.30 ± 3.11), with no significant difference between them. During the late luteal phase, SLCs increased slightly (59.77 ± 3.84), whereas LLCs declined markedly (24.80 ± 2.52). Overall, SLCs counted were highest during the early luteal phase and declined toward the mid phase, while LLCs increased markedly at the mid luteal phase and decreased again at the late phase.

Keywords: Corpus luteum, Histology, Large luteal cell, Small luteal cell

INTRODUCTION

The corpus luteum (CL) is a temporary endocrine structure that develops from the post-ovulatory follicle and is crucial for controlling the estrous cycle and supporting pregnancy by producing progesterone. During its lifespan, the CL undergoes dynamic morphological and functional changes characterized by intense tissue remodeling, including proliferation, differentiation, angiogenesis, and cell death (Neves et al., 2002). These processes are tightly regulated and critical for the establishment and maintenance of a functional luteal structure. The luteal tissue is composed of a heterogeneous population of cells, broadly classified into steroidogenic and non-steroidogenic types (Ponce-Barajas et al., 2023; Fernandez et al., 2024). Endothelial cells, fibroblasts, pericytes, macrophages, mast cells, and leukocytes represent the non-steroidogenic cell population. These cells contribute to structural stability, immune modulation, and vascular support within the tissue. In contrast, the steroidogenic compartment consists of two distinct cell types, including small luteal cells (SLCs) and large luteal cells (LLCs), which differ in their origin, size, morphology, hormone receptor expression, and capacity for steroidogenesis (Shimizu, 2016; Bishop et al., 2022; Fernandez et al., 2024).

Small luteal cells arise from the theca interna layer, are approximately 17 µm in diameter, and are characterized by their irregular nuclear shape, abundant smooth endoplasmic reticulum, and high density of luteinizing hormone (LH) receptors. Although their intrinsic progesterone output is low, SLCs respond acutely to LH stimulation and are involved in the dynamic regulation of luteal steroidogenesis (Ponce-Barajas et al., 2023; Fernandez et al., 2024). Large luteal cells (average ~38 µm), derived primarily from luteinized granulosa cells, are rich in organelles related to steroid production, and are responsible for the majority of progesterone and oxytocin secretion. However, these cells exhibit a weak response to LH because of their low expression of LH receptors (Bertan, 2006; Shimizu, 2016). These cells also store secretory granules containing oxytocin and relaxin and respond to prostaglandin F2α, playing a pivotal role in luteolysis (Skarzynski et al., 2008; Rękawiecki and Kotwica, 2008).

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Although the morphology and function of luteal cells are well characterized, quantitative changes in small and large luteal cell populations across the stages of corpus luteum development have not been fully elucidated. The relative abundance of SLCs and LLCs is known to fluctuate throughout the luteal lifespan, reflecting the gland's changing functional demands. However, previous studies have relied on qualitative or ultrastructural observations rather than direct quantitative comparisons of these cell types at defined stages (Pugliesi et al., 2023). Therefore, the present study provides new quantitative evidence on the relative proportions of SLCs and LLCs in Holstein cows during the early, mid, and late luteal phases, contributing to a clearer understanding of cellular remodeling associated with luteal development and regression. These changes in cellular composition are believed to underlie the shift from hormone-dependent to hormone-independent progesterone production and may influence the luteal lifespan and susceptibility to luteolysis (Basavaraja et al., 2021; Fernandez et al., 2024; Gecaj et al., 2024). Despite this, quantitative histological data on luteal cell populations at defined developmental stages remain limited, underscoring the need for more detailed analysis of luteal tissue organization and function.

Beyond steroidogenesis, luteal cells, particularly LLCs, exhibit significant secretory activity through cytoplasmic granules, which vary in size, density, and composition depending on the physiological stage. These secretory granules contain biologically active peptides, including oxytocin and relaxin, as well as proteins such as tissue inhibitors of metalloproteinases and apolipoproteins involved in extracellular matrix remodeling and lipid metabolism (Skarzynski et al., 2008; Shimizu, 2016; Bishop et al., 2022). The presence of these granules, particularly in LLCs, has been linked to high rates of protein synthesis and endocrine activity. Recent studies have expanded this view, demonstrating that luteal secretory activity is influenced by local immune signaling, angiogenic factors, and metabolic cues that modulate steroidogenesis and cell survival (Miyamoto et al., 2009; Shirasuna et al., 2012; Baddela et al., 2018). Moreover, progressive structural remodeling of the CL-characterized by connective tissue accumulation, altered extracellular matrix composition, and variable vascular density-occurs during luteal regression and advanced pregnancy, reflecting a coordinated balance between synthesis and degradation processes (Junqueira and Carneiro, 2008; Skarzynski et al., 2013).

Despite these advances, comprehensive quantitative data linking cellular composition with functional status remain scarce. The present study addresses this gap by providing quantitative evidence on the relative abundance of SLCs and LLCs across defined luteal phases in Holstein cows. This contributes to a more integrated understanding of how dynamic cellular remodeling supports the transition between active progesterone secretion and structural regression within the bovine corpus luteum (Miyamoto et al., 2009).

Previous studies have investigated the functional and morphological features of luteal cells in various physiological conditions (Xavier et al., 2012; Hryciuk et al., 2021; Ponce-Barajas et al., 2023), but quantitative comparisons of SLC and LLC populations across distinct phases of luteal development, namely early (formation), mid (mature), and late (regression) remain limited. Such data are critical for understanding the shifts in steroidogenic capacity, responsiveness to luteotropic and luteolytic signals, and the cellular mechanisms underlying luteal formation and regression.

Therefore, the objective of this study was to perform a quantitative analysis of small and large luteal cells in the bovine corpus lutea across three key stages of their lifecycle, including post-ovulation formation, functional maturity, and luteal regression. These data aimed to provide a morphological basis for understanding the endocrine functionality of the CL and contribute to the broader knowledge of bovine reproductive physiology.

MATERIALS AND METHODS

Ethical approval

This study involved the use of ovaries collected post-mortem from slaughtered Holstein cows and did not include any live animal handling or experimental intervention. All procedures, including sample collection and analysis, were conducted in accordance with the ethical guidelines for animal research established by the Vietnam National University of Agriculture.

Ovary collection and classification

Ovaries were collected from 30 multiparous Holstein cows (aged 45-90 months) within 30 minutes after routine slaughter at Phuxuyen slaughterhouse, Hanoi, Vietnam. Based on external morphological characteristics, CLs were categorized into three distinct phases (Figure 1) following the criteria described by Miyamoto et al. (2000). In the early luteal phase (formation stage), CLs exhibited a soft texture and a highly vascularized surface, consistent with post-ovulatory development. The mid-luteal phase (mature stage) was characterized by a firm texture and a reddish-yellow appearance, indicative of peak functional activity. In the late luteal phase (regression stage), CLs displayed a pale color and fibrous texture, reflecting structural and functional decline. The sample size was determined using an a priori power

analysis performed with G*Power version 3.1.9.7 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) for a one-way ANOVA (fixed effects, omnibus) design, assuming a significance level (α) of 0.05, a statistical power of 0.80, and an expected effect size (f) of 0.70. The analysis indicated that a minimum of 24 samples (8 per group) would provide sufficient power to detect significant differences among groups. To increase the robustness and reliability of the results, a total of 30 corpora lutea (10 per stage) were included in the study, exceeding the minimum requirement. Classification was conducted by a trained veterinarian using standardized morphological criteria to ensure consistency and minimize observer bias.

Table 1. Number of small and large luteal cells at different stages of corpus luteum (CL) development in cattle (Mean ± SD)

CL Development Stage	Small Luteal Cells (SLC)	Large Luteal Cells (LLC)
Early Luteal Phase (Formation)	72.53 ± 7.83^{am}	30.70 ± 3.78^{an}
Mid Luteal Phase (Mature)	50.70 ± 2.82^{b}	44.30 ± 3.11^{b}
Late Luteal Phase (Regression)	59.77 ± 3.84^{cm}	24.80 ± 2.52^{cn}

^{a,b,c} Different letters indicate differences between different stages of CL development ($p < 0.05$). ^{m,n} Different letters indicate differences between the number of small and large luteal cells ($p < 0.05$). CL: Corpus luteum. The data are presented as the mean ± SD.

Tissue fixation and processing

Following dissection, the CL was carefully separated from the ovary and immediately immersed in 10% neutral buffered formalin for a minimum of four hours to ensure pre-fixation as described by Mokhtar (2015). Subsequently, each CL was sectioned longitudinally into fragments approximately 0.5 cm thick, from the apex (top) to the base (bottom), to preserve the spatial orientation and zonal organization of the luteal tissue. The tissue fragments were then retained in the same fixative solution until further processing.

The tissue fragments underwent dehydration through a graded alcohol series, followed by clearing in xylene and embedding in paraffin using standard histological protocols. Serial sections with a thickness of 5 µm were cut using a rotary microtome (Microm HM 325, Thermo Scientific, Germany), and three representative slides were produced for each corpus luteum. These slides were stained with Hematoxylin and Eosin (H&E) to distinguish nuclear and cytoplasmic components, thereby enabling detailed histological assessment and cellular characterization.

Histological evaluation and cell counting

For quantitative histological analysis, paraffin-embedded sections of the CL were stained with H&E and examined under a light microscope (Japan) at ×40 magnification (Xavier et al., 2012). From each slide, three microscopic fields were randomly selected from different anatomical zones (peripheral, intermediate, and central), yielding a total of nine fields per CL to ensure representative sampling.

Steroid-producing luteal cells were recognized by their distinct morphological characteristics and cell diameters, which were measured using ocular and stage micrometers in accordance with the classification criteria outlined by Yoshioka et al. (2013). SLCs, derived from the theca interna, were characterized by a diameter of less than 20 µm, irregularly shaped nuclei, and dense cytoplasm. In contrast, LLCs, originating from granulosa cells, measured over 35 µm in diameter and exhibited a polygonal shape, abundant cytoplasm, centrally located round nuclei, and prominent nucleoli.

Cell counting was performed within a standardized area of 520 × 340 µm per field. Images were captured using Proview software (Optika Proview, version 4.12.24744, Optika, Ponteranica, Italy) on an Olympus CX31 (Japan) microscope, and the defined counting area was calibrated using an ocular micrometer (Japan). The acquired images were subsequently analyzed using the ImageJ Cell Counter plugin (NIH, USA) to ensure accurate identification and quantification. All counts were performed manually by the same trained observer to reduce inter-observer variability and ensure methodological consistency.

Statistical analysis

Descriptive statistics (mean ± SD) were calculated for each cell type across the three developmental stages. One-way ANOVA was applied to determine statistical differences in cell counts between stages for each cell type. Independent t-tests were used to compare SLC and LLC counts within each stage. Post-hoc analysis using Tukey's HSD test was performed when ANOVA revealed significant results. Statistical significance was set at $p < 0.05$. All analyses were conducted using IBM SPSS for Windows version 22 (IBM Corp., Armonk, New York, United States).

RESULTS AND DISCUSSION

The number of SLCs and LLCs varied significantly across the different stages of CL development (Table 1 and Figure 2). During the early luteal phase, the number of SLCs was highest (72.53 ± 7.83) and significantly greater than that of LLCs (30.70 ± 3.78; $p < 0.05$). In the mid-luteal phase, the number of SLCs decreased significantly compared with the early luteal phase (50.70 ± 2.82 versus 72.53 ± 7.83; $p < 0.05$), while the number of LLCs increased significantly compared with the early phase (44.30 ± 3.11 versus 30.70 ± 3.78; $p < 0.05$). As a result, there was no significant difference between SLCs and LLCs within this stage. This balanced proportion coincides with peak luteal function, characterized by maximal progesterone secretion. The shift toward increased LLCs aligns with previous reports by Shimizu (2016) and Bishop et al. (2022), who noted that LLCs, originating from granulosa cells, possess higher steroidogenic capacity and dominate during the mid-luteal stage when progesterone output is maximal.

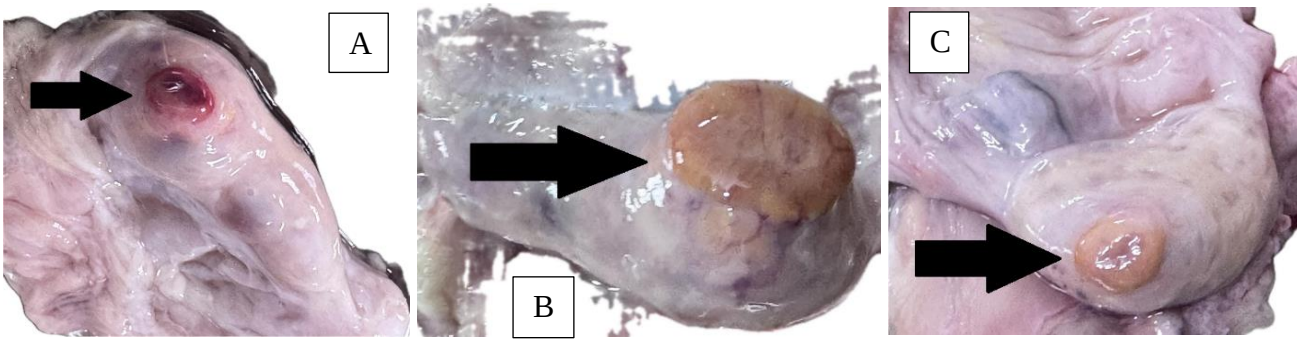
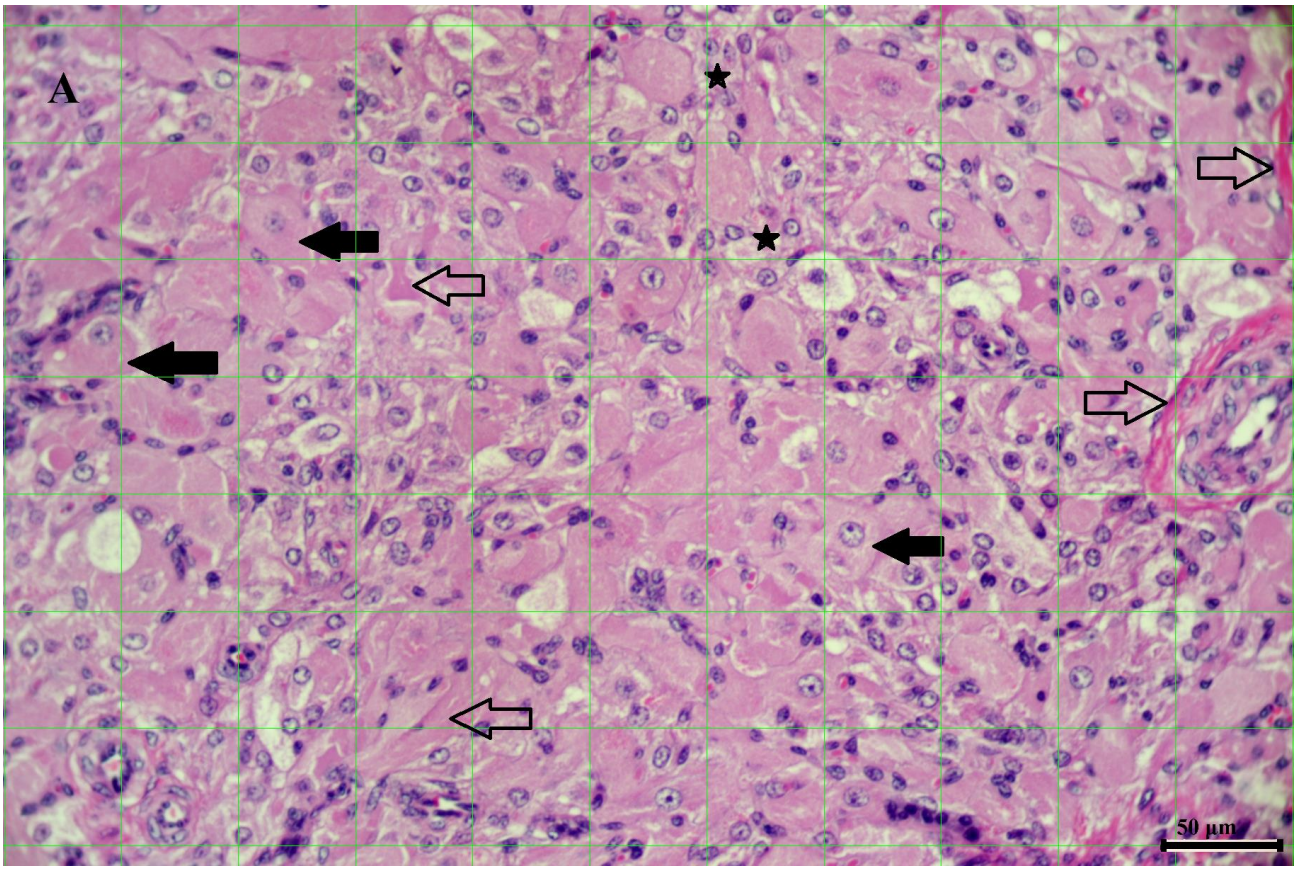


Figure 1. The appearance of bovine corpus luteum at three developmental stages. Representative images of the bovine corpus luteum (CL) at three distinct developmental stages, indicated by black arrows. **(A)** Early luteal phase-newly formed CL post-ovulation, with soft texture and highly vascularized surface; **(B)** Mid luteal phase-mature CL with firm consistency and reddish-yellow coloration; **(C)** Late luteal phase, regressing CL with reduced size, fibrotic appearance, and pale color (Source: Designed by authors).



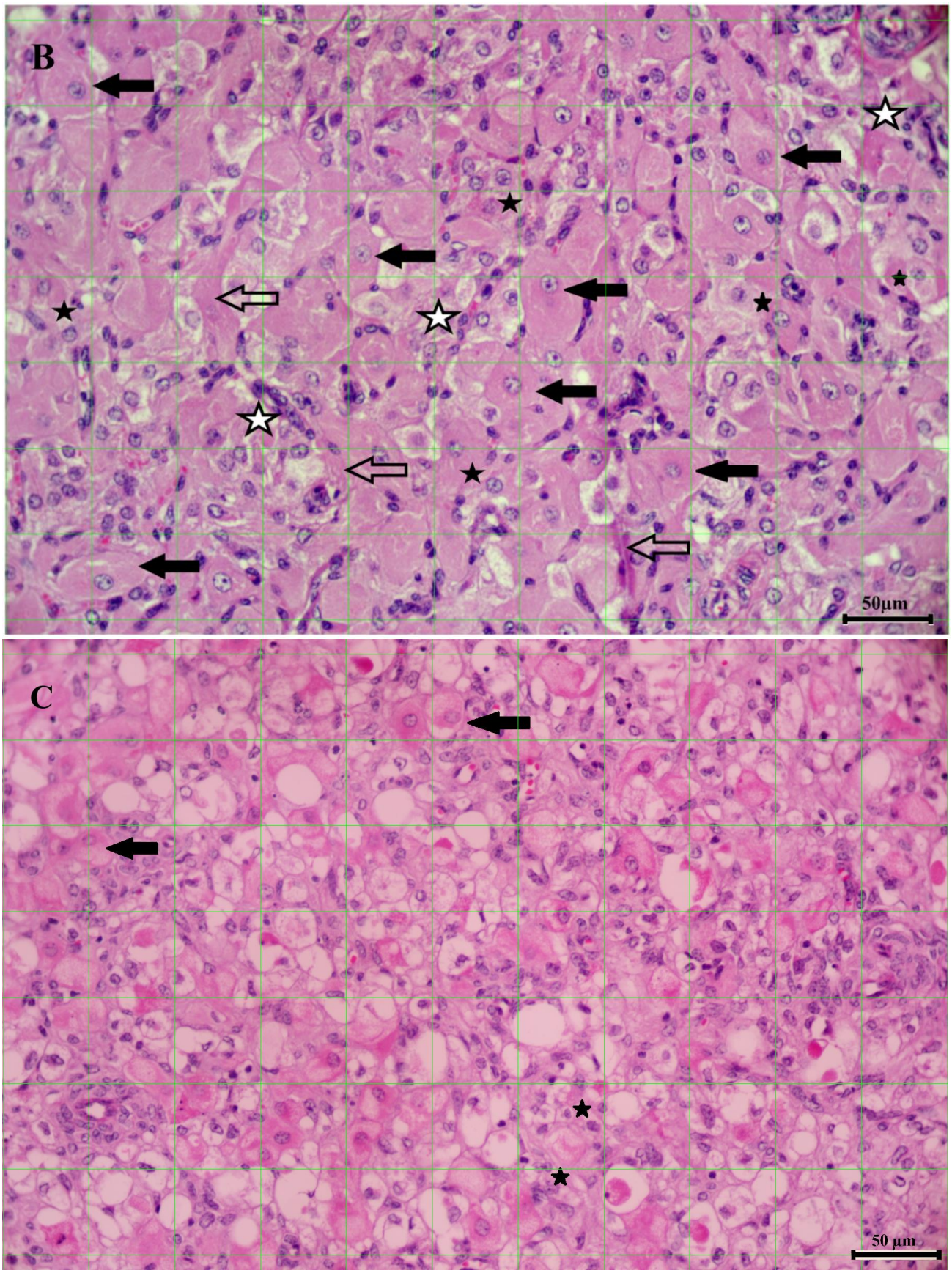


Figure 2. Histological features of the bovine corpus luteum at different luteal phases. (**A**) Early-luteal phase showing newly formed large luteal cells (black arrows) and small luteal cells (black stars) and developing vascular stroma (white arrow). (**B**) Mid-luteal phase showing fully differentiated large (black arrow) and small luteal cells (black star), with well-developed vascular stroma (white arrow) and interstitial fibroblasts (white star). (**C**) Late-luteal phase showing regressive luteal cells (black arrows and black stars), and reduced cellular density. H&E, 40×. The enumeration of large luteal cells and small luteal cells was facilitated using a grid system generated by the ImageJ program. (Source: Designed by authors).

In the late luteal phase, the number of SLCs increased slightly compared with the mid-luteal phase (59.77 ± 3.84 versus 50.70 ± 2.82; $p < 0.05$), whereas the number of LLCs declined markedly compared with the mid-luteal phase (24.80 ± 2.52 versus 44.30 ± 3.11; $p < 0.05$). The decline in LLCs is consistent with luteolytic regression, as LLCs are more sensitive to prostaglandin $F_2\alpha$–induced apoptosis (Shah et al., 2014; Basavaraja et al., 2021). Meanwhile, the relative persistence of SLCs may indicate greater resistance to luteolytic signals, a pattern also observed by Przygrodzka et al. (2021). Collectively, these patterns support the distinct temporal and functional roles of SLCs and LLCs in the cyclic remodeling of the bovine corpus luteum.

This study demonstrated that the number and distribution of SLCs and LLCs vary significantly across stages of CL development in Holstein cows. During the early luteal phase, SLCs predominated, suggesting their active proliferation and involvement in early CL formation, which is consistent with previous reports describing the rapid luteinization of theca-derived cells post-ovulation (Jiang et al., 2011; Ponce-Barajas et al., 2023). In contrast, the mid-luteal phase was characterized by a relatively balanced population of SLCs and LLCs, corresponding to the peak of luteal steroidogenic activity (Shimizu, 2016; Bishop et al., 2022; Fernandez et al., 2024). By the late luteal phase, a significant decline in LLCs was observed, accompanied by a modest increase in SLCs, potentially reflecting early luteolytic changes and the relative resistance of SLCs to apoptosis during CL regression (Przygrodzka et al., 2021). These dynamic changes in luteal cell composition highlight the distinct functional roles of SLCs and LLCs and the tightly regulated structural remodeling of the CL during its development and regression (Bishop et al., 2015; Bishop et al., 2022).

These findings provide new quantitative insights into the dynamic population shifts of small and large luteal cells during the bovine estrous cycle, emphasizing their complementary roles in CL development and function. The predominance of SLCs during the early and developing stages of CL formation indicates that these cells are essential for the initial establishment of the luteal structure and for sustaining early progesterone production before the CL reaches full maturity (Scully et al., 2021; Ponce-Barajas et al., 2023; Fernandez et al., 2024). As the CL progresses to the mid-luteal phase, the relative increase in LLCs, cells with greater steroidogenic capacity, suggests a cellular transition that supports the peak secretion of progesterone necessary for the maintenance of the luteal phase and establishment of early pregnancy (Shimizu, 2016; Bishop et al., 2022). These quantitative relationships between SLC and LLC populations therefore provide a cellular basis for understanding how the CL achieves and regulates its functional capacity throughout the estrous cycle (Bishop et al., 2015; Bishop et al., 2022). By linking morphological cell-type dynamics to physiological function, this study contributes to improving reproductive management and offers a foundation for exploring the cellular mechanisms underlying luteal insufficiency and subfertility in dairy cattle (Diskin and Morris, 2008; Bender et al., 2010; Basavaraja et al., 2021).

The results are consistent with previous findings by Yoshioka et al. (2013), who demonstrated that SLCs show substantial proliferative activity during the early and developing stages, as evidenced by strong Ki-67 expression and the upregulation of cell cycle-related genes such as *CCND2* and *CCNE1*. In contrast, LLCs exhibited minimal or no proliferative activity, supporting the notion that CL growth in the early phase is predominantly driven by SLC proliferation. The predominance of SLCs during early luteal development may be attributed to their origin from theca cells, which retain proliferative potential after ovulation. In contrast, LLCs are derived from granulosa cells that typically undergo terminal differentiation after the LH surge, resulting in cell cycle arrest (Regan et al., 2018; Abedel-Majed et al., 2019). This difference in cellular origin helps explain why SLCs are more numerous and proliferative in the early stages of CL development.

The transition from the developing to the mid-luteal stage is marked by a decline in SLC proliferation and a relative increase in LLC numbers. Yoshioka et al. (2013) demonstrated that LH plays a central role in this transition. LH treatment significantly reduced DNA synthesis and downregulated *CCND2* expression in SLCs derived from mid-stage CLs, but not in those from the developing stage. Concurrently, LH receptor mRNA expression increased at the mid-luteal stage, suggesting heightened LH sensitivity that may promote terminal differentiation and inhibit proliferation of SLCs. These results support the hypothesis that LH signaling mediates a shift from a proliferative to a differentiated state, contributing to the stabilization of luteal cell populations during the mid-luteal phase (Yoshioka et al., 2013). The mid-luteal phase also coincides with peak CL function and high progesterone output, largely due to the abundance and steroidogenic activity of LLCs. Large luteal cells originate from granulosa cells of the preovulatory follicle, and their numbers are closely linked to granulosa cell content. Studies have shown that aspiration of follicular granulosa cells or ovulation of small follicles results in smaller CLs and lower progesterone levels in cows and primates (Vasconcelos et al., 2001; Regan et al., 2018; Abedel-Majed et al., 2019). This indicates that the number of granulosa cells-and thus LLCs-is critical for CL size and steroidogenic capacity.

Molecular data from Hryciuk et al. (2025) further reinforce this relationship. In a controlled luteinized follicular cell culture model, the upregulation of *STAR*, *HSD3B1*, and *CYP11A1*, three essential genes for progesterone biosynthesis, was observed in luteinized granulosa-derived cells. These genes encode proteins involved in key steps of

steroidogenesis, including cholesterol transport (*STAR*), pregnenolone conversion (*CYP11A1*), and the transformation of pregnenolone into progesterone (*HSD3B1*). The observed upregulation in gene and protein expression, coupled with sustained progesterone secretion in culture, indicates that granulosa-derived LLCs can rapidly acquire a steroidogenically active phenotype upon luteinization. Additionally, the downregulation of *CYP17A1*, a key androgen-synthesizing enzyme, during early luteinization suggests a functional shift from androgen to progesterone production, consistent with the luteal phenotype (Magoffin, 2005).

In vivo, LLCs in the mid luteal phase exhibit structural features indicative of high steroidogenic activity, including abundant mitochondria, extensive smooth endoplasmic reticulum, and dense-core secretory granules containing oxytocin and relaxin (Rękawiecki and Kotwica, 2008; Shimizu, 2016; Bishop et al., 2022). These morphological characteristics correlate with increased basal progesterone secretion reported by Bishop et al. (2022) and Fernandez et al. (2024) during the mid-luteal phase, supporting the functional dominance of LLCs at this stage. In addition to LH-driven differentiation, angiogenic and growth factors, along with cell–cell interactions, are likely to support LLC function. Vascular endothelial growth factor (VEGF), which plays a central role in regulating luteal angiogenesis, exhibits elevated mRNA expression during the early and mid-luteal phases, followed by a marked decrease as the corpus luteum undergoes regression (Kisliouk et al., 2007). Additionally, interactions between large and small luteal cells through paracrine signaling have been reported to influence steroidogenic activity within the corpus luteum, as demonstrated in co-culture experiments (Bao et al., 2025).

In contrast, during the late luteal stage, a decline in LLC numbers was observed, coinciding with the initiation of luteolysis. Prostaglandin F2α (PGF2α), the primary luteolytic signal, targets LLCs and induces apoptosis and the release of secretory granules, ultimately leading to a reduction in progesterone secretion (Miyamoto et al., 2009). This selective sensitivity of LLCs to PGF2α is supported by their higher expression of PGF2α receptors compared to SLCs (Basavaraja et al., 2021), which likely accounts for the earlier onset of apoptosis in this cell type. Supporting this, Shah et al. (2014) reported that PGF2α treatment significantly reduces cytoplasmic electron-dense secretory granules in LLCs-but not in SLCs-indicating that degranulation serves as an early and selective indicator of luteolytic activity in LLCs. This persistence may reflect a delayed apoptotic response or reduced sensitivity to PGF2α. However, because of their lower steroidogenic capacity, SLCs contribute minimally to progesterone production during luteal regression (Shimizu, 2016; Bishop et al., 2022). Therefore, the selective loss of LLCs is a critical factor in the decline of luteal progesterone output and functional luteolysis.

Taken together, this data reinforces the model in which the structural and functional heterogeneity of the CL is orchestrated by distinct yet interconnected roles of SLCs and LLCs. These findings contribute to a more comprehensive understanding of luteal cell dynamics and the regulation of progesterone secretion throughout the estrous cycle. Elucidating these mechanisms provides important insight into how changes in luteal cell composition contribute to variations in circulating progesterone levels. Recent studies have highlighted the roles of lipid droplet-associated proteins such as *PLIN2* (Plewes et al., 2024), as well as transcriptional regulators like *STAR* (Zhao et al., 2025), in modulating steroidogenesis and luteal function. Further research should investigate how these molecular factors, particularly those responsive to luteotropic and luteolytic signals, influence luteal cell plasticity and progesterone production. In addition, trace elements such as zinc have been shown to modulate luteal function in a dose-dependent manner by influencing oxidative status and steroidogenic activity (Maruri et al., 2025). Exploring such metabolic and nutritional regulators alongside cellular mechanisms could help identify novel biomarkers or therapeutic strategies to improve reproductive efficiency in cattle.

CONCLUSION

These findings support the concept that the structural and functional heterogeneity of the corpus luteum is governed by the complementary roles of small and large luteal cells. The shifting proportions of SLCs and LLCs across the luteal phases reflect their coordinated contributions to CL development, maintenance, and regression. Future studies should focus on elucidating the molecular mechanisms underlying these cellular transitions, particularly the involvement of lipid droplet-associated proteins, steroidogenic regulators, and micronutrients such as zinc, which has been shown to modulate steroidogenesis and oxidative balance. Integrating cellular, molecular, and metabolic approaches may help identify new biomarkers or therapeutic strategies to enhance luteal function and reproductive efficiency in cattle.

DECLARATIONS

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Authors contributions
Bui Van Dung and Nguyen Hoai Nam conceptualized and designed the research. Data collection and statistical analyses were carried out by Bui Van Dung, Nguyen Thi Thanh Thuy, and Nguyen Thi Ngoc Anh. The initial draft of the manuscript was prepared by Bui Van Dung. All authors participated in data interpretation, provided critical revisions, and approved the final manuscript for publication.

Availability of data and materials
All data and materials supporting the present study are available upon reasonable request from the corresponding author.

Ethical considerations
The authors confirm that this manuscript is original, has not been published previously, and is not currently under review by any other journal. All authors have reviewed and approved the manuscript, ensuring compliance with ethical standards regarding authorship, research integrity, data accuracy, and publication consent. The authors did not use AI in any part of this study including, conducing the study, preparing data, statistical analysis, and writing the manuscript.

Competing of interests
The authors declared no conflict of interest.

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