



Immunohistochemical Study of Steroidogenesis, Proliferation, and Hypoxia-related Proteins in Caprine Corpora Lutea during the Estrous Cycle*

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ABSTRACT : The corpus luteum (CL) is a transient endocrine gland that produces progesterone, a product required for the establishment and maintenance of pregnancy. In the absence of pregnancy, the production of progesterone in the CL decreases and the structure itself regresses in size. The life span and function of the CL are regulated by complex interactions between stimulatory (luteotrophic) and inhibitory (luteolytic) mediators. When an ovum is released from a mature follicle, angiogenesis and rapid growth of follicular cells form the CL. The purpose of the present study was to determine whether steroidogenesis, proliferation, and hypoxia-related proteins are expressed in caprine CL. The expression of proliferating cell nuclear antigen (PCNA), vascular endothelial growth factor (VEGF), and hypoxia-inducible factor 1 α (HIF-1 α) were determined in caprine CL during the estrous cycle. Cytochrome P450 side chain cleavage protein did not vary significantly during the estrous cycle; however, there was an increased expression of 3 β -hydroxysteroid dehydrogenase in the early and middle stages, which rapidly decreased in the late stage. The same observations were made with respect to steroidogenic acute regulatory protein. Variations in progesterone content and expression of PCNA, HIF-1 α , and VEGF were consistent with this result. Thus, the steroidogenic proteins, PCNA, HIF-1 α , and VEGF in caprine CL are dependent on the stage of the estrous cycle. (**Key Words :** Caprine, Corpus Luteum, Steroidogenesis, Hypoxia, Immunohistochemistry)

INTRODUCTION

The corpus luteum (CL) is a transient tissue of female animals during the estrous cycle and pregnancy. The CL's production of progesterone attenuates the pituitary release of gonadotropins and prepares the uterus for a pregnancy. When the ovum is released from a mature follicle during ovulation, angiogenesis and rapid growth of the follicular cells form the CL. The CL is a differentiated follicle in which the theca and granulosa give rise to the small and large steroidogenic luteal cells. As the estrous cycle proceeds, the CL peaks in development, regresses, and then undergoes lysis (Webb et al., 2002; Berisha and Schams, 2005).

The structure and function of luteal cells change during lysis and includes an increase in lipids, vacuolization, lysis

of smooth endoplasmic reticulum, condensation of chromatin, and a rapid decline in progesterone secretion. Above all, a decrease in CL weight and progesterone secretion are important markers of CL lysis. The short and limited life-span of the CL is conducive to the study of the formation and progression of the CL (Knickerbocker et al., 1988; Schams and Berisha, 2002; Madej et al., 2005).

The major product of the CL is progesterone, which regulates the initiation of the estrous cycle and helps maintain pregnancy. To produce progesterone, steroidogenic enzymes are needed; the major enzymes are cytochrome P450 side-chain cleavage (P450_{scc}), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), and steroidogenic acute regulatory (StAR) protein (Rodgers et al., 1986; Suzuki et al., 1993; Tsubota et al., 2001).

The CL is one of the fastest growing and regressing tissue in animals with estrous cycle. Proliferating cell nuclear antigen (PCNA) is an assistant protein to DNA polymerase- δ , and is expressed in the nucleus. PCNA has an important regulatory action during the transition from cell cycle G1 to S phase and it is an important biological indicator of cell proliferation (Christenson and Stouffer,

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1996).

Angiogenesis is a physiologic component of follicular growth and formation of the CL in the ovary during the estrous cycle. Several angiogenic factors that have been described with respect to the ovary; vascular endothelial growth factor (VEGF) plays a critical role in CL angiogenesis (Tscheudschilsuren et al., 2002; Miyamoto et al., 2005; Ferreira-Dias et al., 2006); however, a hypoxic environment is likely involved in angiogenesis during follicular development, selection, and ovulation. It has been reported that hypoxia inducible factor (HIF) mediates adaptive responses, such as angiogenesis, in conditions of low oxygen availability. The regulatory role of HIF in VEGF expression and angiogenesis has been demonstrated in several tumor tissues (Boocock et al., 1995; Watson and Thomson, 1996; Herr et al., 2004). In contrast, no data exists on the expression of hypoxia-inducible factor 1 α (HIF-1 α) protein and its localization in the CL of the caprine ovary (Yamamoto et al., 1997; Boonyaprakob et al., 2005; Miyamoto et al., 2005; Papa et al., 2006).

Therefore, the aim of this study was to investigate the expression and localization of the steroidogenic proteins, PCNA, VEGF, and HIF-1 α in the caprine CL at different stages of the estrous cycle, and to determine the relationship between steroidogenic function and angiogenesis systems in the caprine CL.

MATERIALS AND METHODS

Collection of caprine CL

Care and treatment of all caprine in this study was approved by the Institutional Animal Care and Use Committee of the National Taiwan University. Twenty-six female crossbred Taiwan native goat, 1-3 years of age, were used for the collection of CL. Goats were maintained in a controlled environment. The animals were grown in a sound state of health and demonstrated normal estrous cycle patterns. Two-to-eight animals per stage were used on days 4, 7, 8, 10, 12, 13, and 17 of the estrous cycle. The ovaries were collected immediately after surgery, 2 to 8 CLs were weighed and dissected, and the CLs were fixed in 10% (v/v) phosphate buffer formalin solution (pH 7.4). Blood samples were obtained from each animal daily. Serum was stored at -20°C. Adult females with regular estrous cycles were also checked daily to define the phases of the cycle, as detailed subsequently. Day 1 of the estrous cycle was defined as the first day of low serum progesterone (<2 ng/ml) following the midcycle progesterone surge. Serum progesterone concentrations were measured by enzyme immunoassay. Ovaries were obtained surgically from goats in different luteal phases of spontaneous estrous cycles.

Progesterone assay

The progesterone assay was modified from a direct enzyme immunoassay (Chun et al., 2002; Wu et al., 2002). The G7 monoclonal antibody against progesterone was prepared, as previously described (Wu et al., 2000; Wu et al., 2002). G7 is an IgM with a specific affinity of 1.1×10^{10} . The cross-reactivity of IgM is <0.01% (w/v) with bovine serum albumin (BSA) or other steroids, including pregnenolone, testosterone, estradiol, and estrone.

The serum was stored at -20°C until assayed. Aliquots (50 μ l) of diluted medium and horseradish-peroxidase-linked-progesterone conjugate (150 μ l) were added to microtiter plates coated with 200 μ l of G7 (representing a 1:40,000 dilution). After incubation at room temperature with gentle shaking for 15 min and three washes with Tween-20 in 0.01 M phosphate buffered saline (PBS; pH 7.0) the color was developed with 200 μ l of 3.7 mM O-phenylenediamine in 0.03% (v/v) H₂O₂ for 15 min. The reaction was stopped by the addition of 50 μ l of sulfuric acid. The absorbency of samples, read with a dual wavelength reader at 490/630 nm was compared with that of the progesterone standard curve. The coefficients of variation, within and between assays, were 7 and 12%, respectively. The sensitivity of the assay was 0.3 pg/ml (Wu et al., 2000).

Immunohistochemistry

Samples of caprine CL tissue were fixed overnight in a 10% formalin-fixed solution, embedded in paraffin, and sectioned in 5 μ m pieces. The sections were then mounted on poly-L-lysine-coated glass slides. Following deparaffinizing in xylene, tissue sections were rehydrated by passing them through decreasing concentrations of ethanol to Tris-buffer saline. Deparaffinization was carried out in xylene and was followed by rehydration in a series of graded alcohols at room temperature. Retrieval of antigen binding sites was achieved using 3 \times 5 min citrate buffer (10 mM; pH 6.0), incubated in a microwave oven (100 W). After quenching the activity of endogenous peroxidase with 1% (v/v) H₂O₂ in PBS (pH 7.2) for 10 min, the sections were rinsed (each rinsing was carried out 3 \times 5 min in PBS) and incubated 30 min in 0.02% BSA to reduce non-specific binding. The sections were then incubated with primary antibodies for 24 h at 4°C in a moist chamber. Non-specific binding was blocked with normal caprine serum followed by 3 h incubation with anti-StAR, anti-P450scc, or anti-3 β -HSD antiserum (1: 5,000 dilutions) (Chiu et al., 2008; Chiu et al., 2008). Immunoreactive StAR, P450scc, or 3 β -HSD proteins were visualized with an avidin-biotin-enhanced horseradish peroxidase method (Vector Laboratories, Burlingame, CA, USA) using diaminobenzidine as the substrate, followed by a light nuclear counter-stain with

Table 1. The weights of caprine corpus luteum and ovary on day 4 through day 17 of the natural estrous cycle

Items/n	Estrous cycle (days)						
	4 (n = 2)	7 (n = 4)	8 (n = 2)	10 (n = 2)	12 (n = 8)	13 (n = 2)	17 (n = 6)
Wt. of ovary (g)	1.3±0.08 ^a	1.3±0.20 ^a	1.8±0.33 ^b	1.9±0.55 ^b	2.3±0.53 ^b	2.6±0.63 ^b	1.6±0.72 ^a
Wt. corpus luteum (g)	0.11±0.01 ^a	0.21±0.06 ^b	0.38±0.02 ^c	0.48±0.02 ^d	0.63±0.17 ^d	0.91±0.16 ^e	0.28±0.10 ^b
CL/OV (%)	8.24±0.39 ^a	16.32±3.16 ^b	20.5±0.26 ^b	32.39±3.80 ^c	31.41±5.38 ^c	40.42±0.02 ^d	20.68±7.17 ^b

CL = Corpus luteum; Wt. = Weight; Values are the mean±SEM.

Caprine were primarily crossbred Taiwan native goats from National Taiwan University farm. n: number of animals.

Data point with different letters in the same item is different significantly ($p < 0.05$).

Gill's hematoxylin.

For PCNA identification, clone PC 10 (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody (Waseem and Lane, 1990). For VEGF and HIF-1 α identification, the following antibodies were used, respectively: 1) anti-VEGF is a polyclonal antibody raised in rabbits against a 19 KDa protein (Boocock et al., 1995) and 2) anti-HIF-1 α is a monoclonal antibody raised in mouse clone H1alpha67 against a 120 KDa protein (Semenza et al., 1998). Negative controls for these antibodies were established using goat serum. After incubation with the primary antibody, the sections were rinsed and incubated with biotinylated horse anti-mouse/anti-rabbit secondary antibody for 30 min in a moist chamber. The sections were submitted to another rinsing step before incubation with an avidin/biotinylated horseradish-peroxidase complex (ABC-Method; Vectastain-Universal-ELITEABC-Kit[®]; Vector) for 45 min for signal amplification, according to the protocol of the manufacturer. After undergoing another rinsing procedure, the sections were incubated for 10 min at room temperature with Vector NovaRed[®] (Vector) for visualization of the immunostaining. Finally, the sections were rinsed in distilled water (3×5 min) and counterstained with hematoxylin for 30 s. Tap water

was used for the last rinsing before the slides were mounted with Kaisers Gelatin (Merck; Darmstadt, Germany). Slides were observed under an Axioskop (Zeiss, Germany), equipped with a digital camera (Canon Power Shot A620; Tokyo, Japan). Positive luteal cells for StAR, P450_{scc}, 3 β -HSD, PCNA, VEGF, and HIF-1 α staining were objective in 10 fields per studied animal using 200X magnification.

TUNEL assay

Samples of caprine CL tissues were removed and processed for paraffin embedding. Five micrometers sections were cut and then used for hematoxylin and eosin (HE) staining and TUNEL fluorescence staining. TUNEL staining was performed as described previously (Hara et al., 1998; Sirotkin et al., 2002) using *In situ* Cell Death Detection Kit and DAB reagent (Boehringer Mannheim, GmbH, Mannheim, Germany). Then, the sections were observed using a fluorescence microscope. Cells containing intensive TdT-positive green fluorescence staining in the nuclei were considered apoptotic.

Statistical analysis

All experimental values were expressed as the mean of maximum three repetitions±SEM of data interpreted in all experiments, except immunohistochemistry analysis. Statistical comparisons were performed using ANOVA followed by Duncan's multiple comparison (Duncan, 1995). p values < 0.05 were considered to be statistically significant.

RESULTS

Representative data of the CL shown in Table 1 coincide with ovarian weights, CL weights, and the CL-to-ovary ratio percentage. Most notable for the CL and ovarian weights is the high variation associated with smaller mean values in the newly formed CL (Day 4) and CL undergoing regression (Day 17). This variation was not attributed to differences in the length of cycle, but rather to individual animal differences in the rate of the regressive process.

Serum progesterone concentrations during the estrous cycle in the caprine are shown in Figure 1. These data, based on previously described methodologies revealed that the rapid regression of the CL is the key event upon which

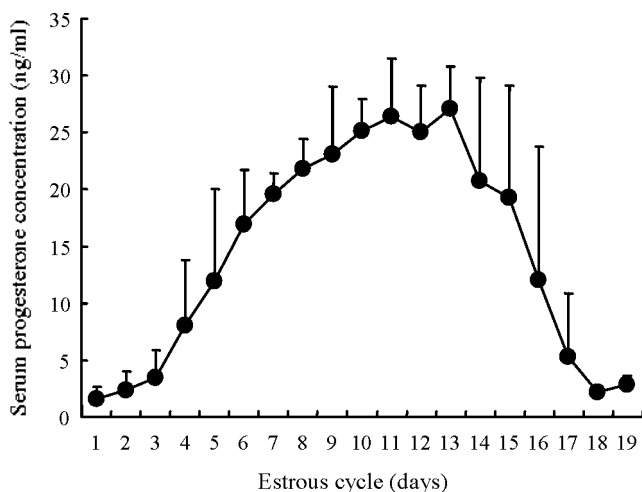


Figure 1. Peripheral serum progesterone concentrations in different days of the estrous cycle in goats. Data are shown as the mean±SEM for nine goats.

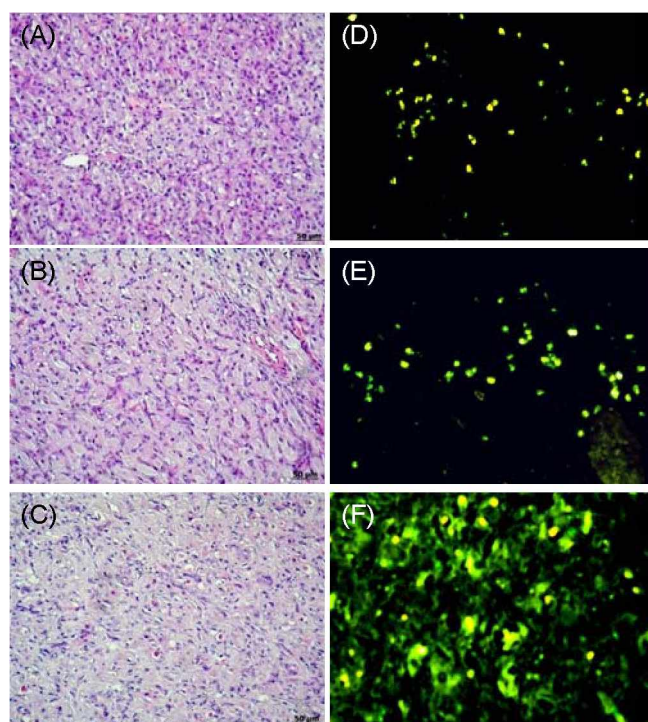


Figure 2. Hematoxylin and eosin staining and TUNEL of caprine corpus luteum in different stages of the estrous cycle. (A),(D) early stage (day 6) CL; (B),(E) middle stage (day 12) CL; (C),(F) late stage (day 18) CL. Bar = 50 µm.

all other cyclical events depend on a decline in circulating progesterone levels.

The CLs were prepared for histologic evaluation at three different stages in the estrous cycle utilizing hematoxylin-eosin (HE) stain techniques and terminal deoxynucleotidyl transferase mediated biotin-dUTP nick-end-labeling (TUNEL) staining (Figure 2). On observation with optical microscopy, we detected a dense structure of the CL tissue with more adherent intercellular junctions in the middle stages than in the early and late stages. As the estrous cycle proceeded, the CL regressed and underwent lysis after reaching the peak in development. The structure and function of the CL cell changed during normal lysis, including an increase in lipids, vacuolization, lysis of smooth endoplasmic reticulum, dissolution of secretory granulosa cells, condensation of chromatin, and a rapid decline in progesterone secretion. Above all, a decrease in CL weight and progesterone secretion were important markers of CL lysis (Fields and Fields, 1996). On observation with optical microscopy, we noted that there was a dense structure of the CL tissue and adherent intercellular junctions in the middle stage than in the early and late stages, but there were more blood vessels in the early stage. The secretion of progesterone was highest during the middle stage. Besides, the identification of apoptotic cells relies chiefly on TUNEL staining. The

sections in different stages were observed using a fluorescence microscope. Either morphological changes (HE staining) or DNA fragmentation (TUNEL method) in the late stage of the CL were detected. A weak nuclear TUNEL positive staining was seen in the early and middle stage of the CL. Cell was identified according to shape and tissue localization. Luteal cells were round, with a large, central nucleus and were present inside the capsule of the CL. Endothelial cells were small and flat, localized around the capillary inner layer. Pericytes were oval in shape and co-localized with endothelial cells surrounding them in the outer layers of the capillaries. Stromal cells were small and localized preferentially among the luteal cells.

The CL produces progesterone and steroidogenic enzymes are needed; the major enzymes are P450_{scc}, 3β-HSD, and StAR protein. Immunohistochemistry results showed P450_{scc} expression, but there was no significant variation during the estrous cycle. However, there was a higher expression of 3β-HSD in the early and middle stages that rapidly decreased in the late stage. The same observations were made with respect to the StAR protein (Figure 3).

PCNA, VEGF, and HIF-1α had the highest expression in the early stage and gradually declined during the estrous cycle (Figure 4), indicating that cell proliferation and angiogenesis rates in the early stages conformed to these results.

DISCUSSION

This study provides an *in vivo* evidence for the existence of steroidogenic proteins (P450_{scc}, StAR and 3β-HSD), proliferation (PCNA), and hypoxia-related proteins (VEGF and HIF-1α) in the caprine CL throughout the estrous cycle. The CL is a transient tissue of female animals during the estrous cycle and pregnancy. As the estrous cycle proceeds, the CL regresses and then undergoes lysis after reaching the peak in development. The structure and function of the CL changes with respect to lysis (Knickerbocker et al., 1988).

A decrease in luteal weight (Table 1) and progesterone secretion (Figure 1) are both important markers of luteal regression. The profile of serum progesterone in goat reflected the steroidogenic capacity of the CL, which paralleled its lifespan. Formation of the CL is initiated by a series of morphologic and biochemical changes in cells of the theca interna and granulosa of the pre-ovulatory follicle. These changes, termed luteinization, occur after the preovulatory LH surge (Schams and Berisha, 2002; Webb et al., 2002). The CL is a heterogeneous tissue consisting of endothelial cells, steroidogenic luteal cells, as well as fibroblasts, smooth muscle cells, and immune cells. In a complex tissue, the various cell types must interact to

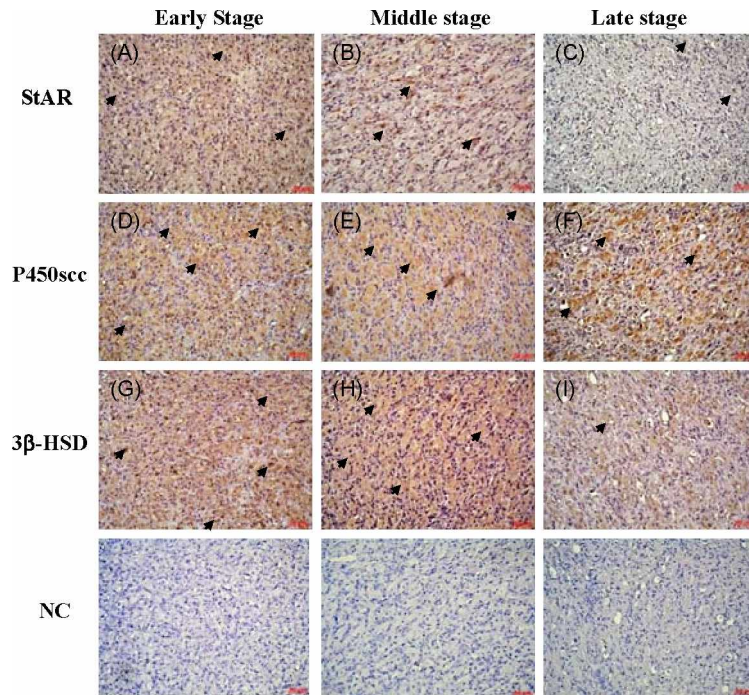


Figure 3. Immunohistochemical localization of StAR, P450scc and 3 β -HSD proteins in different stages of the estrous cycle in caprine corpus luteum. (A), (B), (G) and (H); In early and middle stage immunostaining for StAR and 3 β -HSD were observed in luteal cells (solid arrows). (C) and (I); In late stage, weak immunostaining in luteal cells were observed (solid arrows). (D), (E) and (F); The immunostaining shows P450scc expression did not vary significantly among stages (solid arrows). Early stage CL (day 6); Middle stage (day 12) CL; Late stage (day 18) CL; NC: negative control. Bar = 50 μ m.

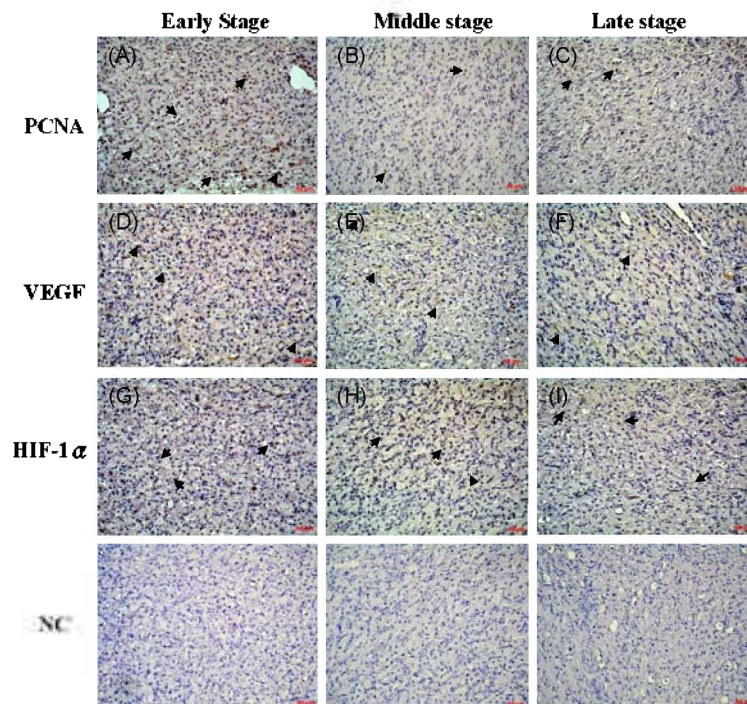


Figure 4. Immunohistochemical localization of PCNA, VEGF and HIF-1 α proteins in different stages of the estrous cycle in caprine corpus luteum. In early and middle stage immunostaining for PCNA, VEGF and HIF-1 α were observed in luteal cells (solid arrows). In late stage, weak immunostaining in luteal cells were observed (solid arrows). Early stage CL (day 6); Middle stage (day 12) CL; Late stage (day 18) CL; NC: negative control. Bar = 50 μ m.

ensure normal growth and development. In this study, we attempted to understand the relationship among progesterone secretion, and expression of P450_{scc}, 3 β -HSD, and StAR protein in different stages of the estrous cycle in caprine.

The CL was surgically collected from goat ovaries in early (days 1-7), middle (days 8-12), and later stages (days 13-21) of the estrous cycle. Using immunohistochemistry techniques, the proteins of P450_{scc}, 3 β -HSD, and StAR in luteal tissue sections were stained with specific antisera produced with specific peptides or recombinant proteins. No significant difference was observed in the P450_{scc} expression among stages. In contrast, the expression of 3 β -HSD and StAR proteins were higher in early and middle stages than in last stages, which correlated well with serum progesterone, gradually elevated to the plateau from the early to the middle stage and decreased in the late stage. The progesterone secretion from the CL directly reflected the correlation between steroidogenic capacity and luteal age. This is the study to show the temporal expression patterns of these three key proteins and the CL in goats (Berisha and Schams, 2005).

CL growth depends on angiogenesis and the establishment of a functional blood supply (Miyamoto et al., 2005). We probed the expression of PCNA, an assistant protein of DNA polymerase- δ , which is expressed in the nucleus, and VEGF, which induces angiogenesis factor and HIF-1 α (Yamamoto et al., 1997; Boonyaparakob et al., 2005). PCNA exerts important regulatory action during the transition from cell cycle G1 to S phase and is an important biological indicator of cell proliferation (Chen et al., 2007; Liu et al., 2007). HIF-1 is a heterodimer of HIF-1 α and HIF-1 β subunits. HIF-1 β is constitutively expressed, whereas the expression of HIF-1 α is maintained at high levels in most cells under hypoxic conditions (Semenza, 1998; Herr et al., 2004; Boonyaparakob et al., 2005). Based on these gene functions, we speculated regarding the relationship between cell proliferation and angiogenesis in the CL during the estrous cycle. When cell proliferation is rapid, the CL undergoes hypoxia and the increase of HIF-1 α may cause expression of the downstream target gene, VEGF. Thus PCNA, VEGF, and HIF-1 α have the highest expression in the early stage, declining gradually during the estrous cycle (Figure 4). Cell proliferation and angiogenesis in the early stage are consistent with these results. The time-dependant protein expression of PCNA, VEGF, and HIF-1 α in the caprine CL described in this study was similar to observations in other domestic ruminants, in which the highest expression of the PCNA and VEGF system was reached in the early stage, followed by a significant decrease in the middle stage and a further decrease during CL regression. That the VEGF system was localized

predominantly in luteal cells was confirmed in the present study for the goat. This study presented a correlative analysis of PCNA, VEGF, and HIF-1 α in different stages of luteal cells and serum progesterone levels, which is indication of CL functional activity. Although functional studies are still needed to better understand the possible influence of VEGF and HIF-1 α on the steroidogenesis in CL of goat, our observation point towards interesting interactions and a possible regulatory role of VEGF and HIF-1 α on caprine in a paracrine and autocrine manner.

In conclusion, our results clearly demonstrated the protein expression of steroidogenesis, proliferation, and hypoxia-related proteins in the caprine CL throughout the estrous cycle. These results suggested that related proteins may play one or more roles in regulating caprine CL function throughout estrous cycle.

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