

Expression of vascular endothelial growth factor and hypoxia-inducible factor-1 alpha during the periovulatory period in goats

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Abstract: To evaluate the expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1 alpha (HIF-1 α) during the periovulatory period, goat ovaries were collected at 0, 4, 8, 12, 24, and 48 h after treatment with 300 IU hCG. Total cellular RNA was isolated and VEGF and HIF-1 α were quantified using real-time RT-PCR. The mRNA levels of VEGF in thecal tissue remained unchanged prior to ovulation. However, the mRNA levels of VEGF in thecal tissues at 48 h increased to 7.5-fold of the level at 0 h ($P < 0.05$). In granulosa tissue, the VEGF mRNA levels were not detected in granulosa cells at 48 h. In thecal tissue, the mRNA level of HIF-1 α at 4 h was 1.5-fold of the level at 0 h and remained unchanged during 8–24 h; however the levels were significantly increased at 48 h. The mRNA levels of HIF-1 α in granulosa at 4 h was not changed but levels were increased ($P < 0.05$) at 8 h and at 12 h and then decreased at 24 and 48 h, respectively. These data describe the relationships of VEGF- and HIF-1 α -dependent angiogenesis during the periovulatory periods by which hypoxia during ovulation is crucial for establishing the thecal new vasculature.

Key words: Vascular endothelial growth factor, hypoxia-inducible factor-1 alpha, periovulatory period, goats

1. Introduction

Vascular growth and development of the preovulatory follicles are mainly controlled by gonadotropins (1) and angiogenic factors (2), especially vascular endothelial growth factor (VEGF). After ovulation, changes occurring include dramatic growth and vascularization of the ovulated follicle transforming it into a corpus luteum (CL). The rate of early luteal development is substantially rapid according to the rapid increase of the preovulatory follicular and luteal vascularity. The CL achieves this vascular supply by recruiting new blood vessels from the thecal-derived vascular beds through the process of angiogenesis (2,3).

Therefore, the periovulatory period provides a paradigm, encompassing the preovulatory follicle to luteal transition, to study the steps involved in the formation of the developing CL (3). Among angiogenic factors, VEGF has potent angiogenic action in the follicle and CL. A ruptured follicle just after ovulation is thought to be under hypoxic conditions because of establishment of new blood

vessels (4). Hypoxia, thus, is important for establishment of vasculature during luteal development and induces hypoxia-inducible factor-1 alpha (HIF-1 α) expression in follicular and luteal cells (5,6).

Hypoxia-inducible factor-1 (HIF-1), consisting of HIF-1 α and -1 β , has been characterized as a transcriptional activator of many oxygen-sensitive genes. During ovulation and early CL formation, VEGF mRNA expression increased significantly under hypoxia, indicating that the hypoxia-induced increase of HIF-1 α regulated the transcription of VEGF (7,8). Although VEGF and HIF-1 α have key roles in angiogenesis in the ovary by which VEGF is upregulated by HIF-1 α under gonadotropin-stimulated conditions, the expression of these angiogenic factors during the periovulatory period remains speculative, except for a handful of species such as sheep (3) and cows (7). Thus, the objective of the present study was to evaluate the expression of VEGF and HIF-1 α genes during the periovulatory period in goats.

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2. Materials and methods

2.1. Animals and collection of periovulatory follicles

All experimental procedures were managed according to the guidelines approved by the Animal Ethics Committee of Khon Kaen University. Prior to experiments, all goats were examined to ensure an absence of reproductive problems and all remained healthy throughout the study. Nulliparous mature female goats ($n = 42$ total; $n = 7$ /group) exhibited at least 2 consecutive estrous cycles and then were stimulated with twice daily intramuscular injections of decreasing dose (21 mg total) of FSH-P (a pituitary extraction; potency of 1.0 Armour units/mg; Sioux Biochemical, Inc., Sioux City, IA, USA) on days 18, 19, and 20. Animals were treated with 300 IU hCG (Chorulon, Intervet, Auckland, New Zealand) on day 20 of the estrous cycle. With this treatment protocol, estrus occurs 12 to 24 h after the last treatment (3,9). Vasectomized goats were used to induce estrous behavior and detect estrus.

Goats were randomly assigned for ovarian tissue collection at 0, 4, 8, 12, 24, and 48 h after hormone injections. Ovaries were placed in ice-cold buffered saline solution and transported to the laboratory less than 30 min after collection. Follicular fluid then was aspirated with a syringe and a 25-gauge, 5/8" hypodermic needle. Thecal and granulosa tissues were obtained from all preovulatory follicles of each ovary. Briefly, thecal tissues were peeled from each follicle with fine forceps whereas granulosa cells were removed from the theca by trituration with a siliconized Pasteur pipette and teasing with forceps. The follicular fluid was centrifuged to recover aspirated granulosa cells. The aspirated granulosa cells were

resuspended in culture medium and the granulosa cells that dispersed into the culture medium were also collected. Tissues were pooled for each goat and snap-frozen for evaluation of VEGF and HIF-1 α expression using real time RT-PCR as described previously (10).

2.2. Estradiol and progesterone assays

Follicular fluid was aspirated from follicles to evaluate estradiol (E2) and progesterone (P4) concentrations in unextracted follicular fluid (diluted 1:100 and 1:20 for E2 and P4, respectively) using commercial ELISA kits as previously described (11,12) with sensitivity of 10.0 pg/mL for E2 and 0.025 ng/mL for P4. The intraassay coefficient of variation for E2 and P4 were 6.25 and 7.75%, respectively.

2.3. Quantitative RT-PCR analysis

Total RNA was isolated separately from the granulosa and thecal tissues by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Addition of polyacrylamide carrier was used to facilitate better yields of from granulosa cells. The RNA quality and quantity were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Bangkok, Thailand). All RNA samples were transcribed in triplicate 20- μ L reactions using the iScript Select cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). All cDNA samples were stored at -20 °C and used in qRT-PCR reactions.

Expression of VEGF and HIF-1 α was studied using the ABI PRISM 7000 Sequence Detection System. Primers and probes were designed to meet the criteria of the software, version 2.0 (Applied Biosystems, Foster City, CA, USA). The details of the primers and fluorogenic probes of genes are shown in Table 1. The qRT-PCR for each cDNA and the 18S rRNA was performed in duplicate and each PCR

Table 1. Target gene, primer, and probe sequences used in the study.

| | Sequences 5'-3' | Efficiency* (%) | Accession number/ reference |
|----------------|--|-----------------|--------------------------------|
| VEGF | Forward: GTG CGG GGG CTG CTG TAA TGA | 98.5 | AY 114352 |
| | Reverse: TCA CCA GGA AAG ACT GAC ACA | | |
| | Probe: (6 FAM)-AAT GCA GAC CAA AGA AAG ATA-(TAMRA) | | |
| HIF-1 α | Forward: AAC GAC AAG AAA AAG ATA AGT | 96.8 | NM 174339 |
| | Reverse: ACC AAG AAG TTG CAT TAA AGT | | |
| | Probe: (6 FAM)-GCT CCT GAG GAA GAA CTA AAT CCA-(TAMRA) | | |
| 18S rRNA | FAM™ | - | X03205.1 |

*: The efficiency of target and reference genes was determined from the concentration curves created using the exogenous control. Cycle threshold value versus the log of starting amount of RNA (ng) was plotted and the slope of the line was determined for each gene. Efficiency was then calculated according to the following equation: Efficiency = $10[-1/\text{slope}]$.

reaction was carried out in a 10- μ L volume using TaqMan Master Mix reagents (Applied Biosystems).

The general real-time PCR protocol was employed for all genes with conditions as follows: denaturation for 30 s at 95 °C, and 40 cycles of a 3-segmented amplification and quantification program (denaturation for 10 s at 95 °C, annealing for 10 s at 58 °C, and elongation for 15 s at 72 °C). For the standard curve, pooled caprine placental RNA from day 65 of gestation was used as a reference, and nuclease-free water was used as a nontemplate control. For an RNA internal control, 18 S rRNA was applied (accession number: X03205.1) and analyzed as described previously (13). Cycle threshold (Ct) values and amplification plots for all genes were analyzed and mRNA for the gene of interest was normalized to 18S rRNA by division of the mRNA values by their corresponding 18S rRNA values and expressed as a multiple of 18S rRNA.

2.4. Statistical analyses

All data are presented as mean \pm SEM. The data were analyzed using analysis of variance (ANOVA) and specific means were evaluated by using Duncan's new multiple

range test (14). Means were considered significantly different if $P < 0.05$, unless otherwise stated.

3. Results

3.1. Number and size of preovulatory follicles

In Table 2, number of preovulatory follicles as well as the average and range of follicular diameters for each time of collection are characterized.

3.2. Estradiol and progesterone concentration in follicular fluid

Compared with 0 h, estradiol concentrations in follicular fluid remained unchanged at 0 and 4 h, decreased at 8 h ($P < 0.05$), and were lowest at 12 to 48 h (Figure 1). Progesterone concentrations also remained unchanged during 0 to 12 h but significantly increased at 24 and 48 h ($P < 0.05$; Figure 2). Thus, the ratios of E2:P4 were greater than 1 from 0 to 12 h and then were less than 1 at 24 and 48 h. The concentrations of E2 and P4 during the periovulatory period in this study corresponded to the profile of E2 and P4 during proestrus and estrus in small ruminants (3,15).

Table 2. Preovulatory follicles characterized in each group.

| Time after hCG injection (h) | Number of preovulatory follicles | Range of follicular size (mm) | Average size (mm) |
|------------------------------|----------------------------------|-------------------------------|-------------------|
| 0 | 5.74 \pm 0.4 | 3–7 | 4.41 \pm 0.2 |
| 4 | 5.86 \pm 0.3 | 2–7 | 5.85 \pm 0.2 |
| 8 | 6.86 \pm 0.4 | 3–8 | 5.80 \pm 0.2 |
| 12 | 7.79 \pm 0.5 | 3–8 | 6.05 \pm 0.3 |
| 24 | 7.30 \pm 0.5 | 3–9 | 6.29 \pm 0.5 |
| 48 | 5.89 \pm 0.4 | 4–8 | 6.68 \pm 0.3 |

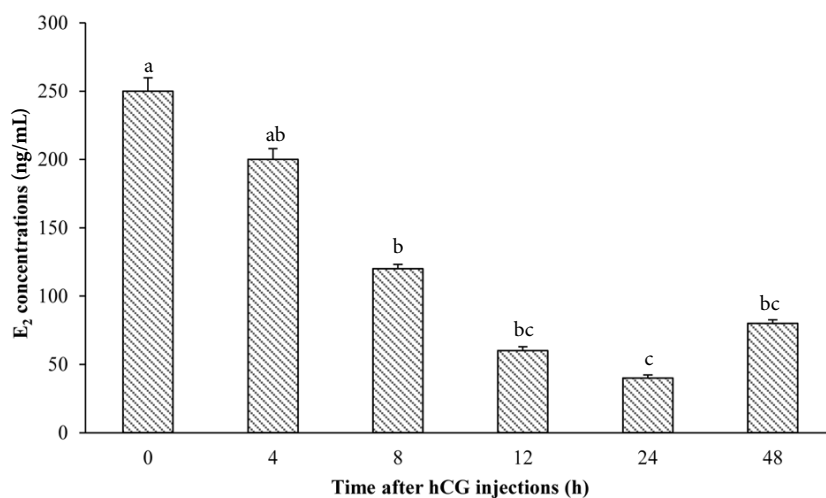


Figure 1. Follicular fluid estradiol concentrations of goats superovulated by FSH and hCG (a, b, and c differ at $P < 0.05$).

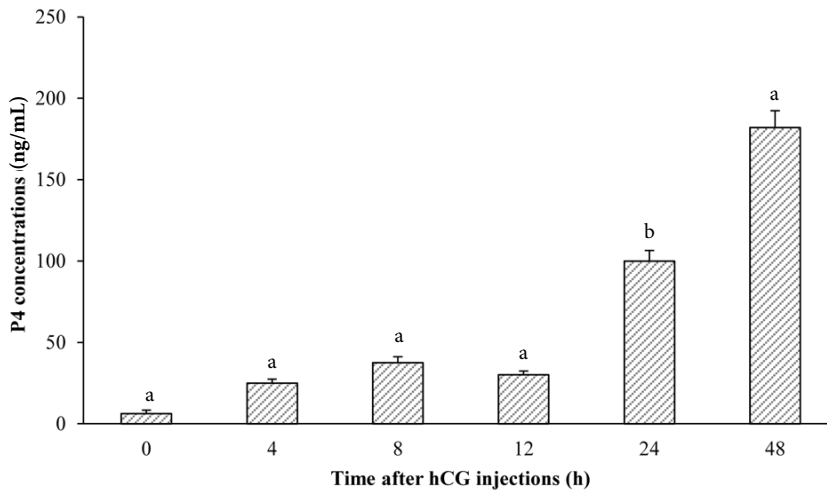


Figure 2. Follicular fluid progesterone concentrations of goats superovulated by FSH and hCG (a, b, and c differ at $P < 0.05$).

3.3. Expression of VEGF mRNA in preovulatory follicles

In the thecal tissues, mRNA levels of VEGF at 4 h increased to approximately 1.5-fold of the levels at 0 h and remained unchanged from 8 to 24 h; however, the mRNA levels of VEGF at 48 h increased ($P < 0.05$; Figure 3) compared to levels at 0 to 24 h by 7.5-fold of the levels at 0 h. In granulosa tissue, the VEGF levels were lower compared to the theca. No increase of VEGF in granulosa was observed during 4 to 8 h, but at 12 h, the mRNA levels of VEGF were greatest ($P < 0.05$; Figure 4) at approximately 2.7-fold of the level at 0 h. The mRNA level decreased significantly at 48 h and was not different when compared with the mRNA levels of VEGF at 4 to 8 h.

3.4. Expression of HIF-1 α mRNA in preovulatory follicles

In the thecal tissues, mRNA levels of HIF-1 α at 4 h increased to approximately 1.5-fold of the levels at 0 h and remained unchanged from 8 to 24 h; however, the mRNA levels of HIF-1 α at 48 h increased significantly ($P < 0.05$; Figure 5) compared to levels at 4 to 24 h at 4.1-fold of the levels at 0 h. The mRNA levels of HIF-1 α in granulosa at 4 and 8 h were similar to the level at 0 h, but levels were increased at 12 h (3.9-fold; $P < 0.05$) and then decreased significantly at 24 and 48 h back to values similar to those at 4 h ($P < 0.05$; Figure 6).

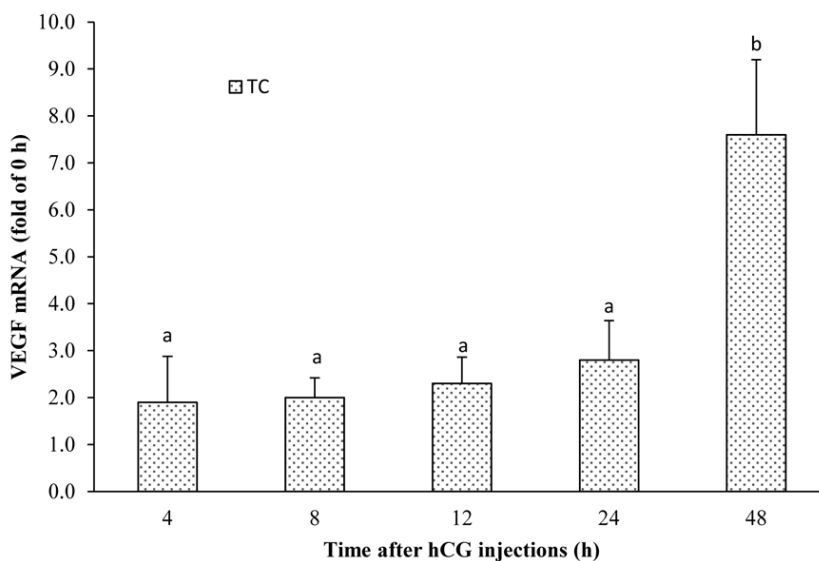


Figure 3. VEGF mRNA expressions (fold of 0 h) in thecal tissue (TC) of goat follicles during periovulatory period (a and b differ at $P < 0.01$).

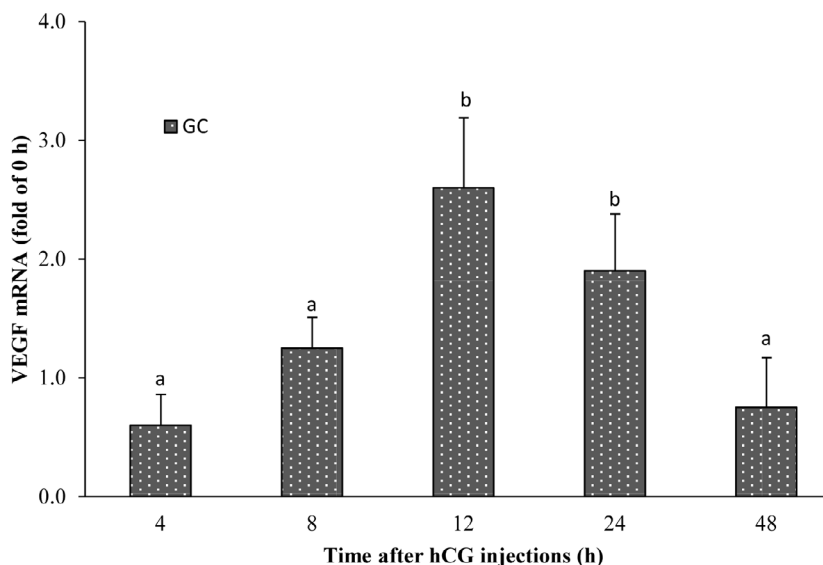


Figure 4. VEGF mRNA expressions (fold of 0 h) in granulosa tissue (GC) of goat follicles during periovulatory period (a and b differ at $P < 0.05$).

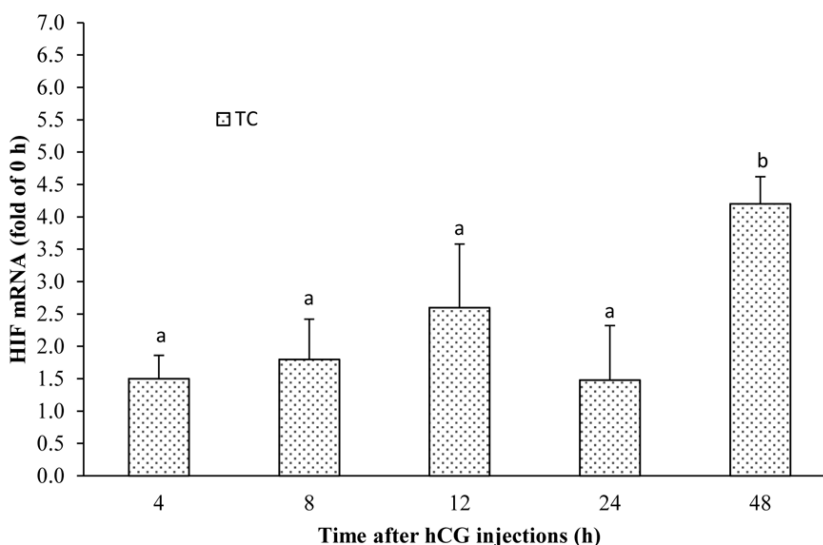


Figure 5. HIF-1α mRNA expressions (fold of 0 h) in thecal tissue (TC) of goat follicles during periovulatory period (a and b differ at $P < 0.05$).

4. Discussion

In this study, we expected that ovulation in goats would occur approximately 24 h after hCG injection (16,17). Tremendous morphological changes were observed by 16–20 h following hCG injection. Development of the preovulatory follicle is associated with increased density of blood vessels within the thecal cell layers surrounding the follicle. Thus, the periovulatory period provides an opportunity to study the steps involved in the transition

of the follicle to the CL and corresponding important angiogenic factors. Among angiogenic factors, a number of studies indicate that VEGF is a potent angiogenic growth factor and is perhaps the most critical regulator of the development of the vascular system (2,18).

Levels of VEGF mRNA in thecal tissue increased immediately at 4 h (about 1.9-fold of 0 h) and then remained unchanged at 8, 12, and 24 h after hCG injection. However, the mRNA levels of VEGF at 48 h

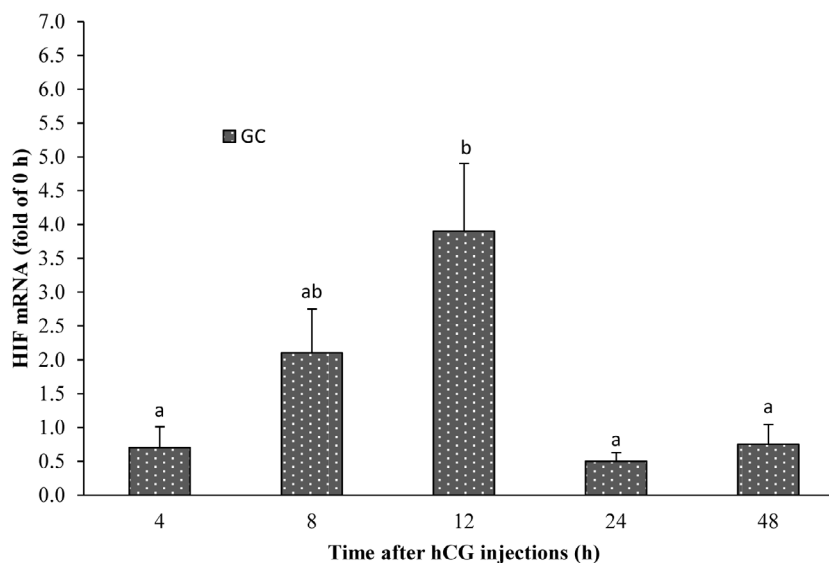


Figure 6. HIF-1 α mRNA expressions (fold of 0 h) in granulosa tissue (GC) of goat follicles during periovulatory period (a and b differ at $P < 0.05$).

increased significantly and were greatest shortly after ovulation. In contrast, differences in VEGF mRNA levels were not detected in granulosa cells, except at 12 (2.5-fold of 0 h) and 24 h (1.9-fold of 0 h) after hCG injection. The present results support those of Redmer et al. (3) that expression of VEGF during the preovulatory period was observed primarily in the theca externa and subsequently in thecal-derived cells that appeared to be invading the granulosa layer during the immediate postovulatory period. Consequently, using immunohistochemistry, no VEGF-positive cells were observed in the granulosa layer of large follicles before the periovulatory period. These observations are consistent with the hypothesis that thecal-derived perivascular cells may direct vascularization of the developing CL via production of VEGF (2). After ovulation, growth and development of new capillaries result in luteal vascularization. This growth of new capillaries follows a cascade of events, including changes in blood vessel basement membrane, migration, and proliferation of endothelial cells, which lead to increased luteal angiogenesis (3). The number of blood vessels per unit area of the luteinizing theca tissue increases as the CL is formed (19). The key step in understanding the entire process of angiogenesis is incumbent upon discerning growth factors that can fulfill all these functional criteria.

The ruptured follicle just after ovulation is thought to be under hypoxic conditions (3,4). The developing CL resembles a healing wound and would be expected to be hypoxic until the luteal parenchymal lobules become well developed and vascularized, and oxygen (O_2) levels would be a major regulator of luteal VEGF expression (4,7). Data

from the goat study support the previous idea that, in sheep, as the follicle matures and approaches ovulation, the granulosa cells produce a factor that stimulates the invasion of pericytes (perivascular cells that are typically associated with endothelial cells in microvessels) into the granulosa cell layer immediately after ovulation (2,3). Pericytes are regulators of endothelial function and have been shown to produce angiogenic factors in vitro (20) under hypoxic conditions and in vivo (3).

Expression of VEGF mRNA in the present study increased dramatically at 48 h. It is likely that gonadotropic ovarian hormones play important roles in the regulation of preovulatory follicular growth and angiogenesis (18,21). Exogenous hCG injection in this study might have upregulated VEGF mRNA expression. These data agree with similar observations by several investigators (22–24) that gonadotropins during the periovulatory period and CL formation regulate VEGF expression. Although other studies have shown VEGF expression by both theca and granulosa layers, we found significant increase only in the thecal layers. These variations in VEGF expression could be due to the different species involved and also due to the different culture conditions as O_2 levels strongly regulate VEGF expression (3,25,26).

The expression of HIF-1 α mRNA in this study increased significantly at 48 h compared to the levels at 4 to 24 h according to the increase of VEGF mRNA, indicating that during the periovulatory period in goat, ovulation causes a decline of local O_2 concentration, providing a hypoxic environment, and may be the main stimulator for VEGF

production in preovulatory follicle and transforming cells for luteal cells (7).

During ovulation, HIF-1 α binds to the promoter region of the VEGF gene and activates its transcription (4,18). Therefore, conditions of O_2 affect VEGF mRNA expression across a number of cell and tissue types, which is consistent with the concept that metabolic demand is the primary factor regulating vascular development in most tissues (2,21). Furthermore, hypoxia-induced increase of HIF-1 α implies that transcriptional regulation of VEGF may be mediated through activation of HIF-1 α because this hypoxia-induced upregulation of VEGF mRNA was suppressed by HIF-1 α inhibition with ferrous ammonium sulfate in luteal cells (4,7). Thus, O_2 appears to be a major regulator of VEGF expression in the bovine (7,8), ovine (3), and caprine ovaries during the periovulatory period.

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In conclusion, these observations describe the relationships of VEGF- and HIF-1 α -dependent angiogenesis during the periovulatory periods in goats by which hypoxia during ovulation is crucial for establishing new vasculature in the thecal issues. However, crosstalk among hypoxia, gonadotropins, and reproductive hormones in regulating VEGF expression during luteal transition needs further investigation.

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