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Stimulatory effect of luteinizing hormone, insulin-like growth factor-1, and epidermal growth factor on vascular endothelial growth factor production in cultured bubaline luteal cells

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ABSTRACT

The purpose of this study was to evaluate the temporal (24, 48, and 72 hours) and dose-dependent (0, 5, 10, and 100 ng/mL of LH, insulin-like growth factor 1 [IGF-1], and EGF) *in vitro* expression and secretion patterns of vascular endothelial growth factor (VEGF) in luteal cell culture during different stages of estrous cycle in water buffaloes. Corpus luteum samples from ovaries of early luteal phase (ELP; Days 1–4), midluteal phase (Days 5–10), and late luteal phase (Days 11–16) were collected from a local slaughterhouse. The samples were then processed and cultured in (serum containing) appropriate cell culture medium and incubated separately with three factors (LH, IGF-1, or EGF) at the previously mentioned three dose–duration combinations. At the end of the respective incubation periods, VEGF was assayed in the spent culture medium by ELISA, whereas the cultured cells were used for VEGF mRNA expression by quantitative real-time polymerase chain reaction. The results of the present study disclosed dose- and time-dependent stimulatory effects of LH, IGF-1, and EGF on VEGF production in bubaline luteal cells. The VEGF expression and secretion from the cultured luteal cells were highest during the ELP, intermediate in the midluteal phase, and lowest in the late luteal phase of the estrous cycle for all the three tested factors. Comparison of the results of the three treatments depicted EGF as the most potent stimulating factor followed by IGF-1 and LH. Immunocytochemistry findings in luteal cell culture of ELP agreed with the VEGF expression and secretion. In conclusion, mRNA expression, protein secretion, and immunolocalization of VEGF data clearly indicated for the first time that LH, IGF-1, and EGF play an important role in stimulating luteal angiogenesis in buffalo CL. The highest expression and secretion of VEGF in the ELP might be associated with the development of blood vessels in early growth of CL, which in turn gets augmented by the aforementioned factors emphasizing their regulatory role in luteal angiogenesis. Further studies are however necessary to divulge more information on other factors which regulate VEGF secretion in bubaline CL and the synergistic effects existing among such growth factors.

1. Introduction

Development of luteal function in mammals requires the formation of new capillary vessels that invade from theca cells of the follicular wall [1,2]. The mature CL is a

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highly vascularized endocrine tissue, and the number of microvascular endothelial cells in this tissue exceeds 50% of the total number of cells [3,4]. Vascular development is a crucial step for formation and maintenance of the CL [5], and therefore, impaired vascular formation may cause luteal insufficiency during the luteal phase or in the early stage of pregnancy.

Among several molecules implicated as mediators of angiogenesis in the CL, vascular endothelial growth factor (VEGF) appears to play a critical role in the regulation of vascular growth, development and function of the CL in several species such as rats [6], marmosets [7,8], humans [9], cattle [10], pigs [11], and buffaloes [12]. The VEGF family comprises seven secreted glycoproteins that are designated VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor, and VEGF-F [13–15]. The VEGFs exert their biologic effects through interaction with their receptors. The receptors identified so far are designated VEGFR-1, VEGFR-2, VEGFR-3, and the neuropilins (NP-1 and NP-2). Vascular endothelial growth factor A is the major regulator of endothelial proliferation and migration [16] and is closely related to the growth of capillary vessels in the CL [11]. Vascular endothelial growth factor A expression in the ovary undergoes dynamic changes during follicle maturation, ovulation, and luteinization, suggesting that expression of this particular local ovarian intermediary factor might be dictated by gonadotropins [17]. Follicle–corpus luteum transition is associated with LH-induced intense angiogenesis [18]. Previous *in vitro* studies have shown that LH and/or hCG and products of its action in target cells are the major regulators of angiogenesis and VEGF-A expression in the primate [17,19], bovine [20], and human [21] ovary, which suggests the role of VEGF as the mediator of gonadotropins in accomplishing luteal angiogenesis. It has been shown that LH, a known activator of adenylate cyclase, induces VEGF messenger RNA (mRNA) expression in cultured bovine granulosa cells [22] and in primate granulosa cells *in vivo* [23].

A dose-dependent stimulatory effect of IGF-1 on VEGF secretion in bovine [20] and human [24] cultured luteal cells has been reported. Massague [25] reported that EGF promotes cell proliferation and is involved in embryogenesis, angiogenesis, and cellular differentiation. Epidermal growth factor receptor expression was evident not only in preovulatory follicles but also throughout the luteal phase [26–30], suggesting that these growth factors might also affect the CL.

Although angiogenesis is distinctly coordinated and well characterized in different functions throughout the body, in the bubaline CL, the molecular mechanism regulating this process is still not fully understood. Therefore, in the present study, we examined the temporal and dose-dependent effects of LH and IGF-1 or EGF on VEGF-A expression in the cultured bubaline luteal cells.

2. Materials and methods

2.1. Establishment of luteal culture system

2.1.1. Coating of coverslips

Coverslips (circular, 12 mm diameter, 0.15 mm thick; Sunbeam, Vyara, India) were soaked in 70% (v:v) industrial methylated spirits for 30 minutes, air dried for 1 hour, and then transferred to a 24-well plate (CLS3524-100EA; Corning, Sigma). Gelatin (0.1%; G1395; Sigma) was added to each well and incubated in a humidified incubator at 38.5 °C. After 4 hours, gelatin was removed and the wells were left to dry at 38.5 °C overnight. On the following day, the wells were washed once with sterile distilled water and allowed to equilibrate with Dulbecco's Modified Eagle's and F-12 Ham's (DME/F12) medium (SH3002301, Hyclone; Thermo Scientific) until cell plating.

2.1.2. Luteal cell culture

Buffalo ovaries were collected from a local abattoir and transported to the laboratory in PBS at 37 °C in a vacuum flask. Corpus luteum samples from the early luteal phase (ELP; Days 1–4; n = 4), midluteal phase (MLP; Days 5–10; n = 4), and late luteal phase (LLP; Days 11–16; n = 4) of the estrous cycle were used for the study, and these were selected on the basis of the criteria outlined previously [16,31]. Briefly, the CL at early luteal stage had hemorrhagic or red colored luteal tissues with loose soft consistency in which blood vessels were not visible. The CL at midluteal stage showed reddish brown to orange color, growing vessels appearing at the periphery and apex with soft to compact consistency. Late luteal stage had tan to orange brown or flesh-colored CL; apex was pinkish with developed vessels at periphery and compact in consistency. On the whole, in total 24 (n = 4/stage × three stages × two repetitions) CLs were obtained and processed for cell culture studies. The experiments were repeated twice for LH, IGF-1, or EGF treatments. Dissected CLs of each type were dissociated according to the technique of Robinson et al. [32] with slight modification. The CLs were removed from the ovary with all connective tissue, blood clot (whenever present) was trimmed away, and then, they were sliced up using BP blades (Bard-Parker Surgical Blade). The minced luteal tissue was washed three times for 5 minutes at 1000 rpm with the culture medium. All cells (including luteal, endothelial, pericytes, and fibroblasts) were dispersed by incubating the luteal tissue in DME/F12 medium containing 2 mg/mL of collagenase 1 type 1A (C-0130; Sigma), 25 μg/mL of DNase 1 (D-5025; Sigma), and 0.5% BSA (A2058; Sigma) for 45 minutes (two times) shaking in an incubating shaker at 37 °C. The dispersed cells from each incubation were pooled together and then filtered through a 70-μm cell strainer (C93070; SPL Life Sciences) to remove nondissociated tissue fragments. The filtrate was then washed twice by centrifugation for 5 minutes at 1000 rpm with DME/F12 media. Erythrolysis was accomplished by washing the pellet with RBC lysis buffer (BWR1003; Biospes Co. Ltd., Chongqing, China). Cell viability was measured using the trypan blue (T8154; Sigma) exclusion test, and the viability was found to be 85% to 90%. The cells were thereafter plated out at 1.5×10^5 viable cells per well in a 24-well plate (total volume: 1-mL media containing 10% fetal bovine serum [F0392; Sigma] and antibiotic and antimycotic solution [Penicillin-G 100 U/mL, Streptomycin 100 μg/mL, Amphotericin 0.25 μg/mL; SV30079.01; Hyclone, Thermo Scientific]) in a humidified CO_2 (5%) incubator at 38.5 °C. The cells were allowed to attach and grow (75%–80% confluent) for 48 hours.