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Stimulatory effect of vascular endothelial growth factor on progesterone production and survivability of cultured bubaline luteal cells

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ABSTRACT

The objectives of the present study were to investigate the effects of vascular endothelial growth factor (VEGF) on progesterone (P4) synthesis in cultured luteal cells from different stages of the estrous cycle and on expression of steroidogenic acute regulatory protein (STARD1), cytochrome P450 cholesterol side chain cleavage (CYP11A1) and 3β-hydroxysteroid dehydrogenase (HSD3B), antiapoptotic gene PCNA, and proapoptotic gene BAX in luteal cells obtained from mid-luteal phase (MLP) of estrous cycle in buffalo. Corpus luteum samples from the early luteal phase (ELP; day 1st–4th; $n=4$), MLP (day 5th–10th; $n=4$), and the late luteal phase (LLP; day 11th–16th; $n=4$) of oestrous cycle were obtained from a slaughterhouse. Luteal cell cultures were treated with VEGF (0, 1, 10 and 100 ng/ml) for 24, 48 and 72 h. Progesterone was assessed by RIA, while mRNA expression was determined by quantitative real-time PCR (qRT-PCR). Results indicated a dose- and time-dependent stimulatory effect of VEGF on P4 synthesis and expression of steroidogenic enzymes. Moreover, VEGF treatment led to an increase in PCNA expression and decrease in BAX expression. In summary, these findings suggest that VEGF acts locally in the bubaline CL to modulate steroid hormone synthesis and cell survivability, which indicates that this factor has an important role as a regulator of CL development and function in buffalo.

1. Introduction

The female reproductive system is an interesting model for the study of angiogenesis in adults because it undergoes a number of programmed angiogenic processes coupled with cyclic evolution and decline of ovarian, endometrial and placental structures (Irustaa et al., 2010). During the course of the estrous cycle (16–21 days in domestic ruminants), luteal tissues exhibit dramatic structural and functional changes including impressive tissue growth, followed by regression, changes in density of vascular bed and progesterone secretion (Jablonka-Shariff et al., 1993; Reynolds et al., 1994, 2000). Gonadotropins and growth factors play a key role in regulating these changes. Vascular endothelial growth factor (VEGF) has various effects on endothelial cells, the most prominent of which is the induction of cell proliferation and differentiation (Gerber et al., 1998a). Several models have demonstrated the angiogenic function of VEGF *in vivo* (Rebar et al., 2002; Gerber and Ferrara, 2003) and *in vitro* (Robinson et al., 2008). However,

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evidence suggests that this growth factor may be involved not only in the control of vascular processes, protecting endothelial cells from apoptosis (Tran et al., 2002), but also in several other steroidogenic and survival functions of distinct cell types (Greenaway et al., 2004; Pfarrer et al., 2006; Kosaka et al., 2007; Gualtieri et al., 2009; Wang et al., 2009; Sousa et al., 2012).

Vascular and non-vascular cellular dynamics during CL establishment culminate in the consolidation of luteal structure and progesterone (P4) secretion. Earlier studies have shown that the inhibition of VEGF *in vivo* during the luteal phase prevents luteal angiogenesis and subsequent progesterone secretion (Wulff et al., 2001; Fraser et al., 2005a, 2006; Duncan et al., 2008). Recent *in vivo* and *in vitro* studies reported a role of VEGF in the modulation of P4 secretion by mid-luteal phase CL of non-human primates (Fraser et al., 2005a) and equines (Galvão et al., 2012). VEGF stimulated progesterone secretion and augmented transcriptional activation of 3betaHSD in equine culture luteal cells (Galvão et al., 2012).

VEGF exerts its biological effects by binding to its respective transmembrane receptors VEGF receptor 1 (Flt-1) and VEGF receptor 2 (Flk-1/KDR), both of which are expressed on endothelial cells. Flk-1/KDR is the principal mediator of the angiogenic effects of VEGF. Flt-1 has limited signaling activity and may act as a decoy or scavenger receptor (Yang et al., 2002), while other studies have implicated Flt-1 in the mediation of endothelial cell proliferation (Zeng et al., 2001) and chemotaxis (Matsumoto et al., 2002). Luteal cell death was associated with decreased local VEGF expression, whilst blockade of VEGF receptors suppressed luteal angiogenesis. These observations indicated possible involvement of VEGF in maintenance of corpus luteum (Geva and Jaffe, 2004; Skarzynski et al., 2008). Moreover, in the fully functional corpus luteum when luteal vasculature is largely complete (Dickson et al., 2001), VEGF seems to act as both endothelial cell survival and vascular permeability factor (Fraser et al., 2005b). VEGF is a strong activator of ERKs (extracellular signal-regulated protein kinases) 1 and 2 via KDR playing a central role in angiogenesis and cell survival (Ilan et al., 1998; Le Couter et al., 2003; Zachary, 2003, 2005; Wang et al., 2009).

The aim of this study was to evaluate the role of VEGF in P4 production in cultured luteal cells from different stages of the estrous cycle and on mRNA expression of STARD1, CYP11A1, HSD3B, classic antiapoptotic (PCNA) and pro-apoptotic (BAX) genes in mid-luteal phase (MLP) of estrous cycle of buffalo.

2. Materials and Methods

2.1. Luteal cell culture

Ovaries were collected from a local abattoir and transported to the laboratory in PBS at 37 °C in a vacuum flask. For the experiments, CL samples from early luteal phase (ELP; day 1st–4th; $n = 4$), mid-luteal phase (MLP; day 5th–10th; $n = 4$) and late luteal phase (LLP; day 11th–16th; $n = 4$) of estrous cycle were used and these were selected based on criteria outlined previously (Chouhan et al., 2013). The CLs were removed from the ovary with all connective tissue and blood clots (whenever present) trimmed away and then sliced up using BP blades. The minced luteal tissue was washed with culture medium three times for 5 min at 1000 rpm. All cells (including luteal, endothelial, pericytes and fibroblasts) were dispersed by incubating the luteal tissue in Dulbecco's modified Eagle and F-12 ham (DME/F12) medium (SH3002301; Hyclone, Thermo Scientific) containing 2 mg/ml collagenase 1 type 1A (C-0130; Sigma), 25 μg/ml DNase 1 (D-5025; Sigma) and 0.5% BSA for 2 × 45 min shaking in an incubated shaker at 37 °C. The dispersed cells obtained after each incubation were pooled together and then filtered through a 70 μm cell strainer (C93070; SPL, Life Sciences) to remove non-dissociated tissue fragments. The filtrate was washed twice by centrifugation for 5 min at 1000 rpm with DMEM/F12 media (Hyclone, Thermo Scientific). Erythrocyte lysis was accomplished by washing the pellet with RBC lysis buffer (BWR1003; Biospes, Chongoing). Cell viability was determined by Trypan blue (T8154; Sigma) dye exclusion test. The cells were then 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 (Sigma) and Antibiotic & Antimycotic solution (Penicillin-G 100U/ml, Strptomycin100 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 (ELP, MLP and LLP) were allowed to attach and grow (75–80% confluent) for 48 h and thereafter the media was replaced with fresh media containing different concentrations (0, 1, 10, 100 ng/ml) of VEGF (human recombinant; lot 0508AF10; Sigma) and incubated for 24, 48 and 72 h. In total, 216 wells were used in the experiment (3 luteal cell phases × 4 doses of VEGF × 3 incubation times × 6 wells per treatment combination). At the end of each specific time duration, the spent culture media from each well were collected and stored at −20 °C until P4 assay, and the harvested cells were used for mRNA isolation.

2.2. Primers

Primers were designed using the Fast PCR (Version: 6.2.73) software. Details of the primers are given in Table 1.

2.3. Quantitative RT-PCR analysis

Total RNA was isolated from different stages of cultured luteal cells by One Step RNA reagent (Bio Basic Inc., Canada) according to the manufacturer's instructions. RNA quality, quantity and integrity were verified by agarose gel electrophoresis and spectrophotometric readings. The integrity of total RNA was checked on 1.0% agarose gel using 1× TBE as electrophoresis buffer. The bands of 28sRNA and 18sRNA reflected the high quality of extracted total RNA. The purity and concentration of total RNA were checked using nanodrop. Isolated RNA samples were free from protein contamination as the OD 260: OD 280 values were more than 1.8. The concentrations of the RNA samples were in the range 200–2000 ng/μl. Total RNA (1 μg) was first treated with 1 U DNase 1 (D-5025; Sigma) at 37 °C for 30 min to digest any contaminating DNA, followed by adding EDTA stop buffer at 65 °C for 10 min. Constant