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Stimulatory effect of vascular endothelial growth factor on Progesterone production, angiogenesis and survivability of cultured bubaline trophoblast cells

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Abstract
To investigate the effects of Vascular endothelial growth factor on progesterone (P_4) synthesis and on the expression of a steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side-chain cleavage (CYP11A1), 3-hydroxysteroid dehydrogenase(3βHSD), proliferating cell nuclear antigen (PCNA), BCL2 Associated X(BAX) and von Willebrand factor (vWF) in trophoblast cells of water buffalo. Trophoblast cell was isolated from the early pregnant uterus. Cells were treated with VEGF @ 1, 10, and 100 ng/ml for 24, 48, and 72h. RIA assessed P4, while mRNA expression by qRT-PCR. P4 secretion was maximum ($P<0.05$) at the highest dose maintained for a longer duration. The mRNA expression of StAR, CYP11A1, 3βHSD, PCNA and vWF were upregulated ($P<0.05$) at 48h and 72h at the highest dose. Expression of BAX was downregulated ($P<0.001$) at 72h. In conclusion, VEGF modulates the TCC on steroidogenesis, cell survivability, and angiogenesis in the placenta through an autocrine and paracrine mechanism.

Keywords: cotyledon, trophoblast cell, vascular endothelial growth factor, progesterone, steroidogenesis

1. Introduction

Proper progression of feto-maternal interactions is critical for the maintenance and development of the fetus during early pregnancy in animals (Verduzco *et al*., 2012) [21]. In cattle, approximately 40% of conception loss is estimated to occur in the period from Day8 to Day16 of pregnancy. Most of the embryonic losses occur during early pregnancy in ruminants (Thatcher *et al*., 1995) [20]. The mammalian placenta is the apposition of fetal cotyledons and maternal caruncles, which play a key role in the physiological exchange between mother and the fetus for maintenance of pregnancy (Mossman, 1937) [8]. Placenta also plays a vital role in the synthesis of steroidogenic enzymes (CYP11A1, StAR and 3βHSD) that regulate cellular and molecular mechanism for the maintenance of early pregnancy in bovine (Verduzco *et al*., 2012) [21]. Steroidogenic acute regulatory protein (StAR), which has been found in gonads, adrenal cortex, and placenta, as well as in the brain of several species regulate the transfer of cholesterol into the mitochondrion for steroid hormones synthesis (Nicol *et al*., 1998; Arensburg, 1999) [9, 1]. Then, cholesterol to pregnenolone is converted by an enzyme cytochrome P45011A1, located on the inner mitochondrial matrix membrane by cleavage (Simpson and Boyd, 1966; Stocco, 2000) [15, 18]. The enzyme hydroxy-d-5-steroid dehydrogenase, 3b- and steroid d-isomerase 1 (HSD3B1) (Pasqualini, 2005) [10]. Subsequently converted pregnenolone to progesterone. Pescador *et al* (1996) [11] reported that StAR, HSD3B1 and CYP11A1 are present in both the caruncle and cotyledon of the bovine placenta during the first 3 months of gestation (Takagi *et al*., 2007) [19].

The Vascular endothelial growth factors (VEGFs) are the most potent inducer of the angiogenic process, vascular permeability, as well as vascular endothelial cell protease production and migration (Reynolds *et al*., 2000) [13]. An in vitro and in vivo study reveals that VEGFs are important growth factors that stimulate angiogenesis, including luteal growth, wound healing, coronary ischemia, and tumour growth (Fraser *et al*., 2000) [5]. VEGF are major angiogenic growth factors of the placenta as these proteins probably account for most of the heparin-binding angiogenic activity produced by both ovarian (Reynolds *et al*., 2000; Babitha *et al*., 2013; Chouhan *et al*., 2013) [13, 2, 4] and placental tissues (Reynolds *et al*., 1987; Zheng *et al*., 1995) [10, 21]. An earlier study of our lab revealed that VEGF modulates steroidogenesis and cell survivability in cultured luteal and granulosa cells in Buffalo (Mishra *et al*, 2016; Chouhan, *et al*., 2014; Mishra *et al*, 2017) [7, 3, 6].

The objectives of the present study were to investigate the effects of VEGF on progesterone (P4) synthesis and on the expression of StAR, CYP11A1, 3βHSD, PCNA, BAX and vWF in trophoblast cells function during early pregnancy in water buffalo.

2. Material and Methods

2.1 Trophoblast cell culture

Trophoblast cell culture model was developed to evaluate the effects of VEGF in trophoblast cell function. Four pregnant uteri (45–60 days of pregnancy) were collected from a local abattoir and transported back to the laboratory in 1X PBS at a 37ºC temperature in a vacuum flask. The uteri were opened, and the placentomes were exposed under laminar flow. The fetal cotyledons of six placentomes per uterus were separated from the maternal caruncle manually. After washing in phosphate buffered saline (1X PBS) for three times, cotyledons were trimmed into small pieces by BP blade in order to obtain a cellular homogenate. The homogenate derived from each animal was pooled, and three disaggregation methods were applied in parallel, mechanical fragmentation with a Teflon mesh (100 grade), enzymatic disaggregation by 0.08% collagenase I type 1A (C-0130; Sigma-Aldrich) for 40 min. After enzymatic digestion, inactivation of the enzymes was carried out by centrifugation. Then, the tissue was washed with Dulbecco's modified eagle medium (Cat#: CC3021.05L; Lot #:34316008; Cell clone, Genetix), and serially filtered through 100 mesh in stainless steel screens (Sigma) to remove undigested tissue. The filtrate was diluted in DMEM/F12 medium (Cat #: CC3021.05L; Lot #:34316008; Cell clone, Genetix); supplemented with 15% FCS (Cell clone, Genetix) and Antibiotic-Antimycotic solution (Penicillin-G 100mg/ml, Streptomycin 100 mg/ml, amphotericin 0.25 mg/ml, SV30079.01; HyClone; Thermo Scientific). The cells consisting of TGC and a low percentage of fibroblastoid cells (<10%) were washed and resuspended in the same culture medium. Cells from each disaggregation method were again pooled and seeded onto polystyrene dishes at an initial density of 8,000,000 cells/ml. Subsequently, the dishes were placed in an incubator at 38.5ºC with 5% CO_2 atmosphere and 100% relative humidity. Trophoblast cells counting was done by haemocytometer and Cell viability was determined by trypan blue exclusion dye (T8154 Sigma-Aldrich) and then1-1.5 x 105 cells per ml were seeded in 12 wells culture plate (total volume: 1 ml containing 15% fetal bovine serum (Sigma- Aldrich) and Antibiotic-Antimycotic solution (penicillin-G 100 IU/ ml, streptomycin 100 mg/ml, amphotericin 0.25 mg/ml, SV30079.01; HyClone; Thermo Scientific). Subsequently, the culture plates were placed in 5% humified CO_2 at 38.5ºC in the incubator. The cells were allowed to attach and grow (70%–75% confluent) for 48 h, and thereafter, the media was replaced with fresh media containing different concentrations (1, 10, and 100 ng/ml) of VEGF and maintained for 24, 48 and 72 h time interval. The doses were selected based on the earlier report (Chouhan, *et al*., 2014; Mishra *et al*., 2016) [3, 7]. Control cells were grown in media without VEGF. Each treatment was tested in triplicate wells in each experiment. At the end of each specific time duration, the spent culture media from each well were collected and stored at −20 ◦C until P_4 assay and the harvested cells were used for mRNA isolation.

2.2 Primers

A set of specific primers were designed from the published sequence and for primer design by DNASTAR software was used. Detail of primer sequence Tm and amplicon length has been presented in Table 1.

Table 1: Target genes, primer sequences (5'□3'), efficiency and amplicon length for q PCR used in this study

Gene	Primer sequences (5'□3')	Efficiency (%)	Amplicon length (bp)	EMBL accession no. or reference
StAR	F: CTGCGTGGATTAACCAGGTTCG R: CCAGCTCTTGGTCGCTGTAGAG	95.1	84	NM_174189.2
CYP11A	F: AGTTCGAGGGATCCTACCCAGA R: AGCCATCACCTCCGTGTTCAG	95.7	146	NM_176644.2
3βHSD	F: GGATCATCTGCCTGTTGGTGGA R: GTGGATGACCACTGAGGTGC	91	191	NM_174343.2
PCNA	F: ACCTGCAGAGCATGGACTCGTC R: CATGCTGGTGAGGTTCACGCCCA	93.3	160	NM_001034494.1
BAX	F: TCTGACGGCAACTTCAACTG R: AAGTAGGAGAGGAGGCCGTC	97.7	250	NM_173894.1
vWF	F: ATCGTAGGGGACTTCCAAGGTGG R: CGGTCTCCAGGTATAGCCCTCTGG	101.9	154	NM_001205308. 1
GAPDH	F: GCGATACTCACTCTTCTACTTTCGA R: TCGTACCAGGAAATGAGCTTGAC	94.7	82	U85042.1

2.3 Quantitative RT-PCR analysis

Total RNA was harvested in triplicate from cells isolated from cotyledons by Qiazol reagent (Cat#: 79306, Qiagen). The integrity was checked on 1.0% agarose gel using 1X tris-borate-EDTA (TBE) as electrophoresis buffer. The bands of 28s RNA and 18s RNA reflected the intactness of extracted total RNA. The purity and concentration of total RNA were checked using nanodrop. Isolated RNA samples were free from the protein contamination as the optical density (OD) 260: 280 values were >1.9. Constant amounts of 1 µg of total RNA were reverse transcribed using cDNA synthesis kit Revert Aid First Strand cDNA Synthesis Kit (Thermo scientific) and oligo-dT18 primer at 42˚C for 60 min. The qPCR for each cDNA and the housekeeping genes GAPDH was performed in duplicate using SsoFast Eva green supermix kit (Bio-Rad) in a Bio-Rad CFX manager Real-Time qPCR as per manufacturer's instructions. The following general qPCR protocol was used: Enzyme activation for the 30s at 95˚C, 40 cycles of a 3-segmented amplification and quantification program (denaturation for 5s at 95 ˚C, annealing for 10s at the primer-specific temperature [55˚C for PCNA, 60˚C for all other examined factors] and elongation for 15s at 72˚C). A melting step was done by slow heating from 61 to 95˚C at 0.58˚C/s and continuous fluorescence measurement, and a

final cooling down to 4˚C. After the end of the run, cycle threshold values and amplification plot for all determined factors were acquired using the ‘‘Eva green (with dissociation curve)” method of the real-time machine (Bio-Rad CFX manager Real-Time QPCR to software) qPCR efficiencies were determined by amplification of a standardized dilution series, and slopes were obtained. The specificity of the desired product was checked using analysis of melting temperature, which is product specific and high-resolution gel electrophoresis to verify that transcripts and confirmed by sequence analysis. Negative control PCR containing all components except template was included for each sample to check out for primer dimer.

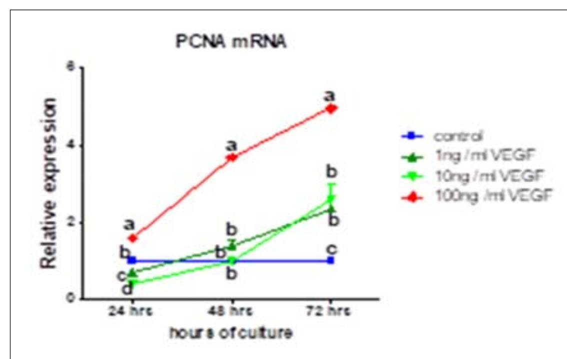
2.4 Gene expression analysis
GAPDH was used as a housekeeping gene. The efficiency of corrected relative quantification of mRNA was obtained by the Pfaffl method (2001) [8]. For this, the efficiency of primers was determined by serial dilution of template cDNA sample, and run in triplicate. The efficiency of the primer of different factors has been given in Table 1.

2.5 Hormone determination
Concentrations of P4 in spent media of TCC was estimated by P4 125I RIA kit (IM1188) supplied by Immunotech, the Czech Republic as per manufacturer's instruction. The spent media was diluted with 1X PBS at 1:5. The measurable range was 0.05 to 50ng/mL for P4. The coefficients of variation were 5.6% and 7.2% respectively for both intra- and interassay.

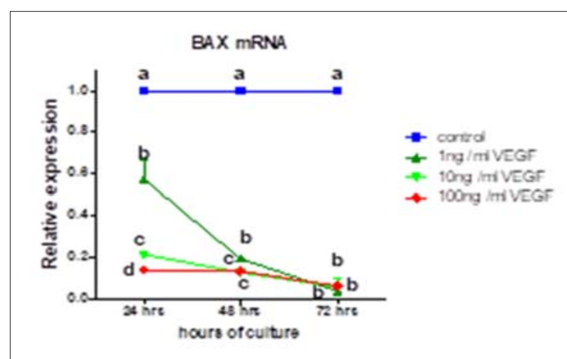
2.6 Statistical analyses
All experimental data are shown as Mean±SEM. The statistical significance of differences in the P4 concentration and mRNA expression of PCNA, BAX, vWF, StAR, CYP11A1 and 3βHSD in cultured trophoblast cells were assessed using the software SAS by two-way analysis of variance followed by Tukey's honestly significant differences test (HSD) as a multiple comparison test. The model included the main effects of the fixed factors (dose of VEGF wherever required) and their expression along with the different time points (24, 48 and72 h). Differences were considered significant if $P<0.05$.

3. Results
3.1 Effect of VEGF on relative mRNA expression of steroidogenesis (StAR, CYP11A1 and 3βHSD) cell survivability (PCNA, BAX) and angiogenesis (vWF) markers in the trophoblast-cultured cell (TCC) *in vitro*
Relative mRNA expression of CYP11A1 (Fig.2C), StAR (Fig.2B) and 3βHSD (Fig.2D) was increased with time of incubation and were maximum ($P<0.05$) during 72h of the cultured period at the highest dose. Relative mRNA expression of PCNA (Fig.1A) was increased between 48h to 72h however, observed highest ($P<0.05$) during 72h of incubation. The BAX expression (Fig.1B) was downregulated from 48h to 72h and found to be lowest ($P<0.001$) during 72h of the culture period. The vWF expression (Fig.1C) was almost similar to PCNA. The relative mRNA expression of vWF, PCNA and 3βHSD were incremented dose and time-dependently ($P <0.05$) in the cells cultured 100 ngml-1 with respect to their corresponding lower doses and control. Therefore, the best dose rate of VEGF was found to be 100 ngml-1.

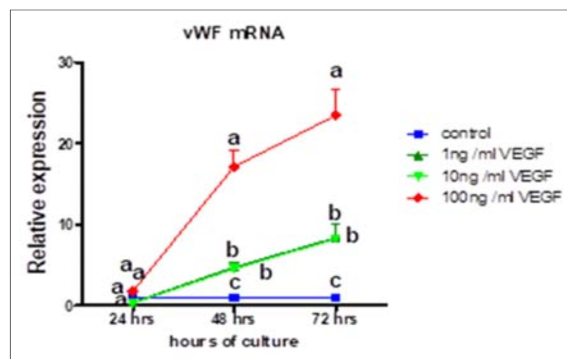
3.2 Effect of treatment with VEGF on the secretion of P4
Hormone estimation from trophoblast cultured cells spent medium which was treated with an increasing dose of VEGF (1, 10 and 100 ng/ml) for 24h, 48h and 72h of incubation was done. P4 concentration with VEGF (Fig.2A) treatment was increased between 48h to 72h however, which was observed highest ($P<0.05$) at 72h of incubation. Higher dose significantly increased production in a dose and time-dependent manner in both treatment and combination.



(A)



(B)



(C)

Fig 1: The different superscripts indicate the concentrations dependent effect at given time point (($P<0.05$). (A) PCNA mRNA in TCC on VEGF treatment; (B) BAX mRNA in TCC on VEGF treatment (C) vWF mRNA in TCC on VEGF treatment; TCC, Trophoblast cell culture; VEGF, Vascular endothelial growth factor; PCNA, Proliferating cell nuclear antigen; BAX, BCL-2 associated X protein; vWF, Von willebrand factor.

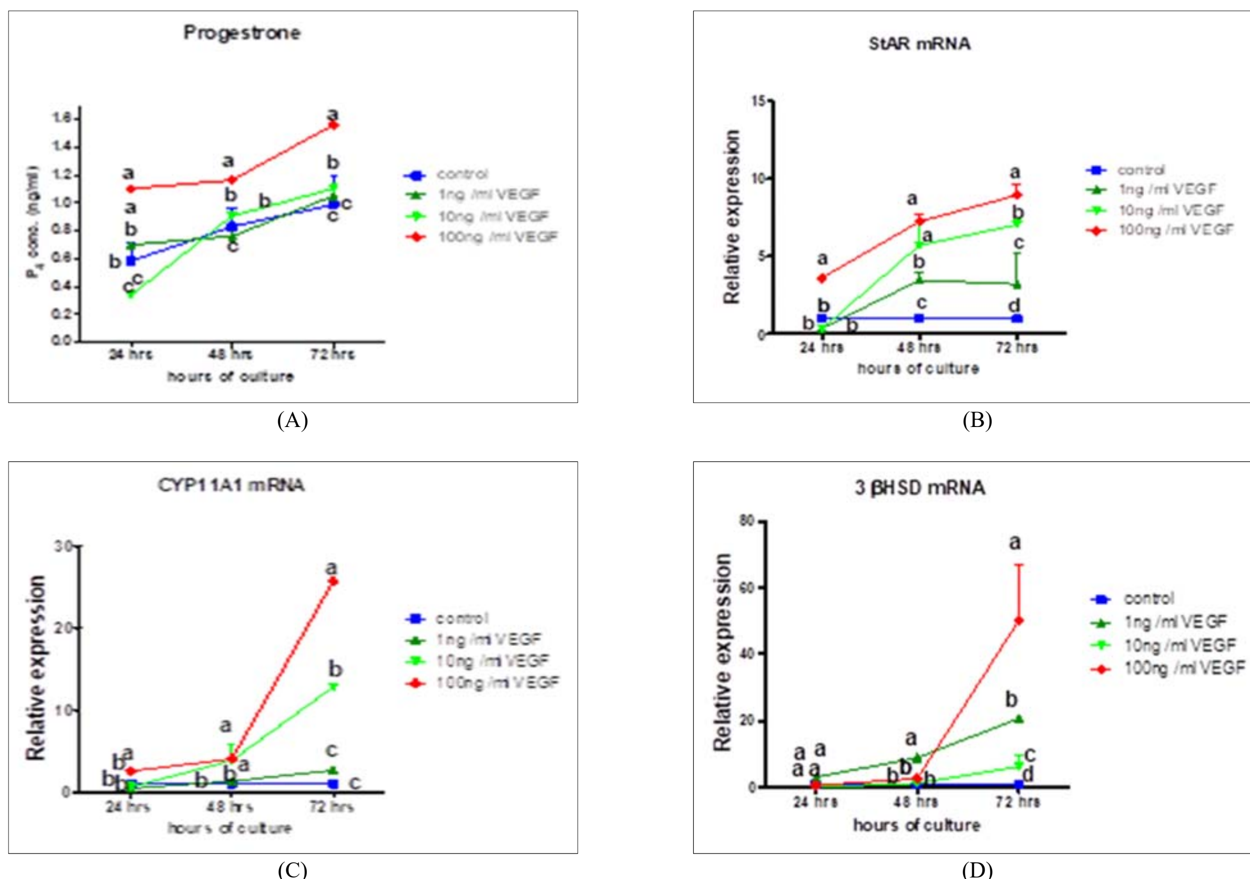


Fig 2: The different superscripts indicate the concentrations dependent effect at given time point ((P<0.05). (A) Progesterone production in the spent cultured medium, (B) StAR mRNA in TCC on VEGF treatment; (C) CYP11A1 mRNA in TCC on VEGF treatment; and (D) 3βHSD mRNA in TCC on VEGF treatment. TCC, Trophoblast cell culture; VEGF, Vascular endothelial growth factor 2; StAR, steroidogenic acute regulatory protein; CYP11A1, cholesterol side chaincleavage enzyme and 3βHSD, 3-beta-hydroxysteroid dehydrogenase

4. Discussion

The present study provides information regarding the buffalo placental steroidogenic, angiogenic and cell survivability capacity during the early stage of pregnancy. The cultured TCC were treated with different doses (1, 10, 100 ng/ml) of VEGF. Out of three doses of VEGF at 100 ng/ml was found to be most effective during 72h of incubation time. The relative mRNA expression of the CYP11A1, StAR, 3βHSD, vWF and PCNA were found to be maximum and BAX expression was minimum in the dose and time-dependent manner. Our results clearly indicated that VEGF at a higher concentration and a longer period of incubation might play a critical role in steroidogenesis mediated by stimulating the expression of steroidogenic enzymes CYP11A1, StAR, and 3βHSD in cultured bubaline TCC. Our results pertaining to CYP11A1, StAR and 3βHSD were in agreement with the earlier report and confirm that placenta contributes significantly during the synthesis of steroid hormones that regulate cellular and molecular mechanism for the maintenance of early pregnancy in bovine (Verduzco *et al*., 2012) [21]. Our data regarding the PCNA and BAX expression most probably suggest the proliferative as well as the anti-apoptotic effect of VEGF on bubaline TCC cultured in vitro which may be due to their mitogenic as well as the cytoprotective role (Zheng *et al*., 1997) [22]. Earlier studies reported that VEGF increased progesterone production in bovine placenta (Sousa *et al*., 2008) [16]. VEGF modulates steroidogenesis in the bovine placenta during gestation in a different pattern in vitro (Sousa *et al*., 2012) [17].

Our results clearly indicated that VEGF at a higher concentration and a longer period of incubation might play a critical role in steroidogenesis mediated by stimulating the expression of steroidogenic enzymes CYP11A1, StAR, and 3βHSD in cultured bubaline TCC. Our data regarding the PCNA, BAX and vWF expression might suggest the proliferative as well as anti-apoptotic and angiogenesis effect of VEGF on bubaline TCC cultured *in vitro*.

5. Conclusion

In conclusion, our data clearly suggests that VEGF modulates in TCC steroidogenesis, cell survivability, and angiogenesis in the bubaline placenta through an autocrine and paracrine mechanism.

Conflict of Interest

The authors declare that there is no conflict of interest on publishing the article.

6. Acknowledgments

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