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Expression and localization of fibroblast growth factor (FGF) family in corpus luteum during different stages of estrous cycle and synergistic role of FGF2 and vascular endothelial growth factor (VEGF) on steroidogenesis, angiogenesis and survivability of cultured buffalo luteal cells

S.R. Mishra [a], M.S. Parmar [a], V.S. Chouhan [a], G. Rajesh [a], V.P. Yadav [a], M.K. Bharti [a], Jaya Bharati [a], T. Mondal [a], R. Reshma [a], A. Paul [a], S.S. Dangi [a], B.C. Das [b], L.A. González [c], G.T. Sharma [a], G. Singh [a], M. Sarkar [a,*]

[a] Division of Physiology & Climatology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh 243122, India
[b] ERS, Indian Veterinary Research Institute, Izatnagar, Kolkata 700 03, India
[c] Centre for Carbon, Water and Food, School of Life and Environmental Sciences, The University of Sydney, Camden, NSW, Australia

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ABSTRACT

The aim of this study was to document the expression and localization of fibroblast growth factor (FGF) family members comprising of fibroblast growth factor (FGF1, FGF2, FGF7, FGF10), and their receptors (FGFR1, FGFR2, FGFR3, FGFR4, FGFR2IIIB, FGFR2IIIC) in buffalo corpus luteum (CL) obtained at different stages of the estrous cycle. In addition, the synergistic role of FGF2 and/or vascular endothelial growth factor (VEGF) on P_4 secretion and mRNA expression of steroidogenic acute regulatory protein (StAR), cytochrome P450 (CYP11A1), 3-beta-hydroxysteroid dehydrogenase (3βHSD), proliferating cell nuclear antigen (PCNA), BCL-2 associated X protein (BAX) and von willebrand factor (vWF) were studied in luteal cell culture obtained from mid-luteal phase (MLP) of estrous cycle in buffalo. Real-time PCR (qPCR), western blot, and immunohistochemistry were used to investigate mRNA and protein expressions, and the localization of examined factors whereas P_4 secretion was assessed by RIA. The mRNA and protein expression of FGF1 and FGFR1 were maximum ($P < 0.05$) during MLP whereas FGF2 was maximum ($P < 0.05$) during early luteal phase (ELP). FGF7, FGF10, FGFR2, FGFR3, FGFR4, FGFR2IIIb, and FGFR2IIIc mRNA and protein expression did not change among luteal phases. FGF family members were localized in cytoplasm of luteal cells as well as in endothelial cells. P_4 secretion in luteal cells treated with FGF2 or VEGF alone showed the maximum values ($P < 0.05$) with the highest dose at 72 h. P_4 secretion was found to be greater ($P < 0.05$) in luteal cells treated with FGF2 + VEGF compared to FGF2 or at 72 h of incubation. The mRNA expression of all factors were maximum ($P < 0.05$) whereas BAX was minimum ($P < 0.05$) at highest dose cultured for 72 h of luteal cells subjected with either protein alone or in combination. Summarizing, the present findings explore the synergistic role of FGF2 and VEGF on steroidogenesis, angiogenesis, cell viability through an autocrine and paracrine actions in buffalo CL.

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1. Introduction

Water buffalo (*Bubalus bubalis*) is an important livestock species found mostly in India and developing countries of Asia. Indian buffaloes are mostly reared for milk, meat, draft purpose, and play an important role in the Indian economy. Indian buffaloes contribute about 68% of the total milk production in the world (FAOSTAT, 2012). Between 30 and 40% of the buffaloes remain unproductive which is, in part, due to the reproductive problems such as subestrus, anestrus, and infertility, thus incurring an estimated loss of 19–20 million tonnes of milk each year (Madan and Prakash, 2007). Normal luteal cell function requires a continuous process of angiogenesis (Schams and Berisha, 2004; Miyamoto et al., 2010; Robinson, 2013) whereas inadequate vascularization of corpus

Abbreviations: Fibroblast growth factor, FGF; Vascular endothelial growth factor, VEGF; Corpus luteum, CL; Progesterone, P_4; Fibroblast growth factor receptor, FGFR; Steroidogenic acute regulatory protein, StAR; Cytochrome P45011A1, CYP11A1; 3-beta-hydroxysteroid dehydrogenase, 3Bhsd; Proliferating cell nuclear antigen, PCNA; BCL-2 associated X protein, BAX; Von willebrand factor, vWF; Pre-ovulatory follicles, PF; Quantitative real time-polymerase chain reaction, qRT-PCR; Complementary DNA, cDNA; β microglobulin, β2M; Ribosomal protein S15a, RPS15A; polyvinylidene-difluoride, PVDF; Bovine serum albumin, BSA; Horseradish peroxidase, HRP; 4′, 6 -diamidino-2-phenylindole dihydrochloride, DAPI; Luteal cell culture, LLC; Fluorescein isothiocyanate, FITC; Glyceraldehyde 3-phosphate dehydrogenase, GAPDH.

* Corresponding author.
E-mail address: msarkar24@gmail.com (M. Sarkar).

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luteum (CL) results in a decrease in progesterone (P_4) secretion which ultimately leads to poor embryo development and infertility (Hazzard and Stouffer, 2000; Chouhan et al., 2013; Wiles et al., 2014). Ovarian abnormalities impair the CL development which leads to failure of pregnancy (Ali et al., 2009; Nam and Aiumlamai, 2010), while luteal cell dysfunction is the major cause of early embryonic mortality in buffalo (Campanile and Neglia, 2007). Therefore, identifying and understanding of the endocrine, autocrine, and paracrine mechanisms of luteal angiogenesis that regulates the luteal development and function would help reducing luteal cell dysfunction and luteal related infertility, which could improve reproductive efficiency of buffalo.

The CL is a temporary endocrine gland that secrets large amount of P_4 essential for survival of embryo, implantation, and maintenance of pregnancy (Niswender et al., 2000; Miyamoto et al., 2010; Robinson, 2013). The development and function of CL require a complex interaction of multiple angiogenic factors including FGF, VEGF (Chouhan et al., 2014a), TGF-β, IGF (Uniyal et al., 2015), EGF(Chouhan et al., 2014b, Chouhan et al., 2015), PGDF and angiopoietin (Schams and Berisha, 2004; Kaczmarek et al., 2005; Otrock et al., 2007; Robinson et al., 2009). The fibroblast growth factor (FGF) family consists of 22 different signalling peptides, which mediate their biological activities through high affinity trans-membrane tyrosine kinase receptors known as fibroblast growth factor receptor (FGFR) (Sleeman et al., 2001; Itoh and Ornitz, 2004). At the best of times FGFs activate several receptors by binding to FGFR1–4 along with some splice variants of FGFR2, *i.e.* FGFR2IIIB and FGFR2IIIC (Powers et al., 2000; Berisha et al., 2004; Bottcher and Niehrs, 2005). FGFs are involved in the growth, development, function, and regression of the CL regulating ovarian function in different species including bovine (Gospodarowicz et al., 1985; Schams et al., 1994; Parrott and Skinner, 1998; Salli et al., 1998; Berisha, 2001; Nilsson et al., 2001; Guerra et al., 2008).

FGF2 plays an important role the in initiation of angiogenesis during early periods of luteal development, whereas VEGF stimulates angiogenesis during luteal development in bovine CL (Woad et al., 2012). Moreover, neutralization of FGF2 by injecting FGF2 antibody leads to a reduction in luteal growth and steroidogenesis in bovine CL (Yamashita et al., 2008) whereas continuous inhibition of either FGF2 or VEGF down regulates luteal ECs network formation (Woad et al., 2009). The combination of FGF2 and VEGF induced a greater angiogenesis than either growth factor alone *in-vitro* (Pepper et al., 1992), which was confirmed with *in vivo* studies (Asahara et al., 1995) and both *in-vitro* and *in vivo* conditions (Kano et al., 2005).

Based on these previous evidences, FGFs and VEGF are the principal regulators in luteal cell angiogenesis and steroidogenesis process. However, to the best of our knowledge, no information is available in regards to the synergistic effect between FGF2 and VEGF in buffalo CL. Therefore, the objectives of the present study were to investigate: (1) mRNA and protein expression along with the immunohistochemical localization of FGF family members (FGF1, FGF2, FGF7, FGF10, FGFR1, FGFR2, FGFR3, FGFR4, FGFR2IIIB, and FGFR2IIIC) in buffalo CL during different stages of the estrous cycle, (2) the effects of FGF2 and VEGF on P_4 secretion in luteal cell culture (LCC) and, (3) the effects of FGF2 and VEGF alone, and a combination of both on mRNA expression and localization of major steroidogenic pathway intermediates (StAR, steroidogenic acute regulatory protein; CYP11A1, cytochrome P45011A1; 3βHSD, 3-beta-hydroxysteroid dehydrogenase), classic cellular proliferation marker (proliferating cell nuclear antigen, PCNA), pro-apoptotic marker (BCL-2 associated X protein, BAX), and endothelial cell marker (von willebrand factor, vWF) in cultured bubaline luteal cells.

2. Materials and methods

2.1. Collection of CL during the estrous cycle

Apparently normal morbid reproductive tract of buffalo cows were collected immediately after exsanguination at a local abattoir and transported on ice pack to the laboratory. The Stages of the estrous cycle were estimated by macroscopic observation of the ovaries (Kumar et al., 2012). Forty ovaries (n = 10 CL/group) were used for total RNA isolation, western blotting and immunohistochemistry studies. CLs were classified as early luteal phase (ELP; days 1–4), mid luteal phase (MLP; days 5–10), late luteal phase (LLP; days 11–16), and regressed CL (days >16) of estrous cycle. Luteal tissue was frozen in liquid nitrogen and stored at −80 °C until RNA and protein isolation.

2.2. Collection of follicles during final follicular growth

Only follicles (n = 10) which appeared healthy (*i.e.*, well vascularized and having transparent follicular wall and fluid and whose diameters were >14 mm were used. Large follicles (>14 mm) were collected only after CL regression, with signs of mucus production in the uterus and cervix and were assumed to be preovulatory. For RNA extraction, follicles were dissected from the ovary. The surrounding tissue (theca externa) was removed with forceps under a stereomicroscope as previously described (Babitha et al., 2013). FF aspirated from follicles was stored at −20 °C until determination of P_4. As healthy follicles have relatively constant P_4 levels in FF, only follicles with P_4 < 100 ng/mL FF were used for the evaluation to exclude atretic follicles (Kumar et al., 2012). Follicles were snap-frozen in liquid nitrogen and stored at −80 °C until RNA and protein isolation.

2.3. Hormone determination by RIA

P_4 concentration in the FF and spent culture media of LCC were estimated by P_4 ^{125}I RIA kit (Cat No#IM1188, Immunotech, Czech Republic) as per manufacturer's instruction. The measurable ranges were 0.05 ng/mL to 50 ng/mL. The intra and inter assay coefficient of variations were 6.5% and 8.1% respectively.

2.4. Primers

All the Primers were designed using the DNASTAR-lasergene (version 6) software. Primers used in this study were synthesized by Eurofins Genomics, Karnataka, India. The details of the primers and primer efficiencies are presented in Table 1.

2.5. Quantitative RT-PCR

Total RNA was isolated from CL by using TRIzol reagent (Invitrogen) according to manufacturer's instructions. Total RNA was treated with DNase-1 (Invitrogen) to exclude genomic DNA contamination. Constant amounts of 1 μg of total RNA from CL (n = 10 per group) were reverse transcribed to complementary DNA (cDNA) by using iScript™Select cDNA Synthesis Kit (Cat No#170-8891, Bio-Rad Laboratories, USA) at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min as per manufacturers' instructions. The cDNA was used as template in qRT-PCR study. The qRT-PCR was performed in duplicate by using SsoFast™ EvaGreen® Supermix kit (Cat No#172-5201AP, Bio-Rad Laboratories, USA) in Stratagene MX3005P qPCR System (Agilent technologies) as per manufacturers' instructions. A total reaction volume of 10 uL (0.5 uL of cDNA + 0.25 uL forward primer (0.25 μM) + 0.25 uL reverse primer (0.25 μM) + 5 uL of SsoFast™ EvaGreen® Supermix + 4 uL PCR graded nuclease free water) was subjected to qPCR study as per standard protocol. The two step qPCR protocol was following: segment 1 (single cycle, enzyme activation - 95 °C for 30 s), segment 2 (40 cycles, denaturation - 95 °C for 5 s, annealing/extension - 60 °C for 10 s), a melting step by slow heating from 62 °C to 95 °C with a rate of 0.58 °C/s with a continuous fluorescence measurement, and final cooling down at 4 °C. No template control (NTC) or negative control with all components except template was done for each sample to rule out the formation of primer dimers. Standard curve to check the primer efficiencies were determined by amplification of a standardized dilution series of cDNA. The

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