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Research paper

Expression and functional role of Bone Morphogenetic Proteins (BMPs) in cyclical corpus luteum in buffalo (*Bubalus bubalis*)

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ABSTRACT

The role of growth factors in the modulation of ovarian function is an interesting area of research in reproductive biology. Recently, we have shown the expression and role of IGF, EGF, VEGF and FGF in the follicle and CL. Here, we report the presence of Bone Morphogenetic Proteins (BMPs) and their functional receptors in the corpus luteum (CL) of buffalo. The bubaline CL was classified into four stages according to the morphology and progesterone (P_4) concentration. The qPCR, immunoblot and immunohistochemistry studies revealed that BMP2 and BMP Receptors (BMPR1A, BMPR1B and BMPR2) were significantly upregulated during the mid stage whereas BMP4 and BMP7 were upregulated during the early stage of CL ($P < 0.05$). Studies on primary luteal cell culture (LCC) using mid CL showed a significant time and concentration dependent effect of BMP4 and BMP7 ($P < 0.05$). At 100 ng ml^{-1}, the BMPs maximally stimulated the transcripts of StAR, CYP11A1 and 3βHSD that paralleled with P_4 accretion in the media ($P < 0.05$). Further, the BMP4 as well as BMP7 upregulated the transcripts of PCNA and downregulated CASPASE3 in the LCC at the same concentration ($P < 0.05$). Though the combined effect of BMP4 and 7 was significantly higher ($P < 0.05$) than that of individual one, it was not additive. In conclusion, the expression of BMPs and their receptors were dependent on the stages of CL in the buffalo. Treatment of LCC with BMPs *in vitro* confirmed the presence of functional receptors that stimulated the P_4 production and luteal cell survival. Moreover, the results support the concept that the upregulation of P_4 and its biosynthetic pathway enzymes such as CYP11A1, StAR and 3βHSD in the CL is likely due to the autocrine and /or paracrine effects of BMP4 and BMP7 under physiological milieu.

1. Introduction

The water buffalo (*Bubalus bubalis*) is an economically important food animal and has its ecological niche in the Asia. It is a versatile species and is used as milch, meat and draft animal. Despite, the production potential of buffalo is yet to be realized, which is, in part, due to the inherent reproductive problems such as delayed puberty, silent estrus, seasonal breeding, long postpartum ovarian inactivity and variable response to superovulation (Madan and Prakash, 2007). The estrous cycle of the buffalo is akin to that of white cattle. For instance, the follicular growth occurs in a wave like pattern and the length of luteal phase is similar to that of cow. Pituitary derived gonadotropins and growth hormone regulate the final follicular maturation and corpus luteum (CL) function through the intraovarian growth factors (Babitha et al., 2013; Gupta et al., 2014). In fact, locally produced growth factors modulate the recruitment, selection and dominance of the follicle in ovulation (Fortune et al., 1988) and luteogenesis, luteostasis and luteolysis in the cow (Berisha and Schams, 2005; Hyashi et al., 2003) and in the buffalo (Babitha et al., 2014; Gupta et al., 2014; Singh et al., 2015; Uniyal et al., 2014). Regulation of luteinizing hormone (LH) receptor expression and its function coupling with LH are the intraluteal events likely to be controlled by the growth factors. Thus, a better understanding of the autocrine and/or paracrine factors that regulate the CL function in terms of P_4 secretion and the enzymes involved in its production have potential clinical significance for aberrations of CL function.

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A plethora of growth factors that belongs to the transforming growth factor β (TGF-β) superfamily, are expressed by ovarian somatic cells and oocytes in a developmental, stage-related manner and function as intraovarian regulators of folliculogenesis (Knight and Glister, 2001, 2006; McNatty et al., 2002; Shimasaki et al., 2004). Bone morphogenetic proteins (BMPs) are a family of multifunctional growth factors that belongs to TGF-β superfamily of proteins. The biological actions of BMPs are mediated through a hetero-oligomeric complex of the BMP type 1 (BMPR1A and BMPR1B) and type 2 (BMPR2) receptors. This complex allows phosphorylation of type 1 receptors by type 2 receptor, which subsequently leads to transphosphorylation of intracytoplasmic signaling molecules, SMAD1, 5, and 8 (Massague, 1998; Miyazono, 2000; Nohe et al., 2004; Yang et al., 2015). Originally discovered for their ability to induce bone formation, BMPs are now known to play crucial roles in all organ systems. In human, over 20 members of BMPs are shown to be involved in cell proliferation, differentiation, embryogenesis, neurogenesis, hematopoiesis and apoptosis (Carreira et al., 2014; Kayamori et al., 2009).

Studies on BMP and its receptor system in the ovary are emerging. The mRNAs for BMP2, BMP4, BMP6, BMP7 and the type IA, IB, and II BMP receptors are expressed in a tissue specific manner in the ovary of rat (Shimasaki et al., 1999), cow (Glister et al., 2010) and ewe (Juengel et al., 2006; Souza et al., 2002). The BMPs function as luteinization inhibitors by suppressing the LH receptor expression in GC of rat (Shimasaki et al., 2004). Additionally, the BMP system is shown to play a crucial role in folliculogenesis in the bovine (Glister et al., 2010). Of the BMPs, BMP7 is highly expressed in the theca cell layer of the ovarian follicles (Shimasaki et al., 2004). BMP4 and BMP7, the theca cell derived growth factors directly affect the GC function (Kayamori et al., 2009; Lee et al., 2001; Shimizu et al., 2012b). Moreover, BMP4 and BMP7 suppress the apoptosis of GC (Kayamori et al., 2009; Shimizu et al., 2012a).

Given their role in ovarian follicular function, we hypothesized that BMP and its receptors are expressed in the CL and regulate the luteal cells in the buffalo. Accordingly, the present study was done with the following objectives: A) To evaluate the transcriptional, translational and immunohistochemical profile of BMP and its receptors in bubaline CL during different stages of the estrous cycle. B) To study the effects of BMP4 and/or BMP7 on Progesterone (P_4) secretion, StAR, CYP11A1 and 3βHSD mRNA expression in the primary culture of luteal cells. C) To study the effects of BMP4 and/or BMP7 on antiapototic PCNA and proapoptotic CASPASE3 mRNA expression in the primary culture of luteal cells.

2. Materials and methods

2.1. Collection of CL

Apparently normal morbid genitalia of buffalo cows were collected at a local slaughterhouse within 10–20 min after exsanguinations and were transported on ice to the laboratory. The stage of the estrous cycle was defined by macroscopic observation of the ovaries (color, consistency, CL stage, number, and size of follicles) and the uterus (color, consistency, and mucus) as described previously (Sarkar et al., 2010). Forty ovaries, each with CL, were used to extract 10 CL per group for RNA extraction, immunoblotting, and immunohistochemistry studies. The CL was assigned to the following stages: early luteal stage (days 1–4), mid luteal stage (days 5–10), late luteal stage (days 11–16), and regressed CL (days >17) of estrous cycle. Luteal tissue was frozen in liquid nitrogen and stored at −80 °C until RNA and protein isolation (Kumar et al., 2012).

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 follicle (PF). For RNA extraction, follicles were dissected from the

Table 1
Target gene, primer sequences and amplicon length used in the qPCR study.

Gene	Sequence of nucleotide (5′-3′)	Efficiency (%)	Amplicon length (bp)	EMBL accession No. or reference
BMP2	Forward: AAGGCCCTTGCTTGTCACTTT Reverse: TGCTTGCCGCTTTTCTCTTC	102.3	73	NM_001099141.1
BMP4	Forward: TTTATGAGGTTATGAAGCCCCCGGC Reverse: AGTTTCCCACCGCGTCACATTGTG	104.2	104	NM_001045877.1
BMP7	Forward: GGCAGGACTGGATCATCG Reverse: GAGCACAGAGATGGCATTGA	99.7	191	NM_001206015.1
BMPR1A	Forward: TCAGCGAACTATTGCCAAACAG Reverse: CCCATCCACACTTCTCCGTATC	103.6	75	NM_001076800.1
BMPR1B	Forward: TGGATGTCTAGGACTAGAAGGCTC Reverse: CAAAATCTCTGTTTTTCAGCGGA	98.9	149	NM_001 1 05328.1
BMPR2	Forward: AACACCACTCAGTCCACCTC Reverse: GTCAGCATCCTATATCCAAAGCA	100.7	120	NM_001304285.1
3βHSD	Forward: GATCATCTGCCTGTTGGTGGA Reverse: GTGGATGACCACTGAGGTGC	98.2	191	Kumar et al. (2012)
CYP11A1	Forward: AGTTCGAGGGATCCTACCCAGA Reverse: AGCCATCACCTCCGTGTTCAG	110	146	Kumar et al. (2012)
StAR	Forward: CTGCGTGGATTAACCAGGTTCG Reverse: CCAGCTCTTGGTCGCTGTAGAG	97.1	84	Kumar et al. (2012)
PCNA	Forward: ACCTGCAGAGCATGGACTCGTC Reverse: CATGCTGGTGAGGTTCACGCCCA	98.6	160	NM_001034494.1
CASPASE3	Forward: CAGGGTGCCCAGGACTTTAG Reverse: AGAAAGCTCACGGGAACCAG	97.3	165	NM_001077840. 1
RPS15A	Forward: AGGGCTGGGAAAATTGTTGTGAA Reverse: TGAGGGGATGGGAGCAGGTTAT	104.8	125	Mishra et al. (2015)
Beta actin	Forward: AGTTCGCCATGGATGATGA Reverse: TGCCGGAGCCGTTGT	104.4	54	Singh et al. (2015)

Abbreviations: **BMP**, Bone Morphogenetic Protein; **3βHSD**, 3beta hydroxysteroid dehydrogenase; **CYP11A1,** Cytochrome P450 family 11 subfamily A member 1; **StAR**, Steroidogenic acute regulatory protein; **PCNA**, Proliferating cell nuclear antigen; **CASPASE3**, Cysteine aspartic acid protease3; EMBL, European molecular biology laboratory.