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EXPRESSION AND LOCALIZATION OF BONE MORPHOGENETIC PROTEIN6 (BMP6) IN THE CORPUS LUTEUM DURING DIFFERENT STAGES OF ESTROUS CYCLE IN THE BUFFALO (*Bubalus bubalis*)

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KEYWORDS

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ABSTRACT

Emerging evidence suggests that Bone Morphogenetic Proteins (BMPs), which belong to the Transforming Growth Factor-β (TGF-β) super-family, are known to be involved in the follicular growth and steroid production in different species. This study describes BMP6 bearing in corpus luteum over various stages of estrous cycle. The bubaline CL was classified into four stages according to the morphology and progesterone (P_4) concentration. The real time PCR and immunoblot studies revealed that BMP6 was significantly ($P<0.05$) unregulated during the mid stage of CL that was consistent with immunohistochemical localization in the luteal cells. The transcriptional and translational expressions of BMP6 in the early and late CL were comparable and significantly ($P<0.05$) lower than that of mid CL. In conclusion, BMP6 expression is dependent on the stage of CL in the buffalo.

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1 Introduction

Livestock production constitutes a very important element of the agricultural economy of developing nations. In India, milk, meat and draft purposes is fulfilled by water buffalos (*Bubalus bubalis*) and act as an important livestock for rural community. Buffalo milk shares 62% of total national milk production in India (DAHDF, 2012). According to FAOSTAT (2012), there are 194.2 million heads of buffalo in the world, of which 115.4 million is in India (59.4% of total world population). High milk yield with more fat content, good feed conversion efficiencies, comparatively higher disease resistant than the bovines are the peculiarities of the buffaloes (Kumar et al., 2012). Owing to the versatility of buffaloes, they are called 'black gold' by the farmers (Bilal et al., 2006). However, the buffalo is considered to be a poor breeder due to high incidence of delayed puberty, silent estrus, summer anestrus and long postpartum interval (Barile, 2005; Madan & Prakash, 2007). Anomalies in the angiogenesis and vascularization of the corpus luteum (CL) may result in premature luteal regression, decreased progesterone (P_4) production and unexplained infertility (Madan & Prakash, 2007). Hence, there is a need to study the role of intraluteal autocrine and /or paracrine factors with respect to luteogenesis, luteostasis and luteolysis.

It is known that the luteal function is under the negative feedback control of hypothalamo-hypophyseal ovarian axis. But, it is well established that growth factors produced locally had crucial permissive and modulatory function in growth of follicles, ovulation, dominance and luteal function (Fortune et al., 1988; Hyashi et al., 2003; Berisha & Schams, 2005). These local factors include Transforming growth factor-β (TGF-β),Vascular endothelial growth factor (VEGF), Insulin-like growth factor (IGF), Fibroblast growth factor (FGF), Epidermal Growth Factor (EGF) and angiopoietin etc. (Ferrara et al., 2003; Kaczmarek et al., 2005; Bramley et al., 2005; Otrock et al., 2007). Among the ovarian factors, members of the TGF-β super family have a biological role in the folliculogenesis in the cow (Knight & Glister, 2006; Glister et al., 2010).

Bone morphogenetic proteins (BMPs) are categorized under the TGF-β super family. BMPs are a group of bioactive proteins that were originally isolated from the extract of bone matrix of by Urist in 1965. To date, over 20 BMPs have been identified and shown to be involved in the regulation of cell proliferation, survival, differentiation and apoptosis, chondrogenesis, osteogenesis and embryogenesis. BMPs exhibit their actions by stimulating the membrane attached threonine/serine kinase receptors. The BMPs transduce the signals *via* the classical BMPs–Receptor–Smads signal pathway (Nohe et al., 2004). Recently, BMPs have attracted much attention in the field of ovarian physiology. It is known that the BMP receptor mRNAs are present in the ovary, with the strongest expression in the granulosa cell (GC) and in the oocyte, which is consistent with the BMP's action observed on the GCs (Shimasaki et al., 1999; Wilson et al., 2001).

The BMPs function as luteinization inhibitors by suppressing luteinizing hormone (LH) receptor expression in the GCs (Shimasaki et al., 2004). Additionally, the BMP system was shown to play a crucial role in folliculogenesis in humans (Shi et al., 2009; Shi et al., 2010; Shi et al., 2011). Of the BMPs, BMP6 is highly expressed in the theca cell layer in the ovarian follicles and CL of rat (Shimasaki et al., 2004) and cow (Glister et al., 2010). Until now, no published information is available regarding BMP6 expression and localization in various stages of development of CL in buffalos. Hence, the present study was focused on the mRNA and protein expression along with the immunohistochemical localisation of BMP6 in the CL during different stages of the estrous cycle in the buffalo.

2 Materials and Methods

2.1 Collection of corpus luteum

Buffalo cow genetalias which are apparently normal were collected from the local slaughter houses and transported with the help of ice to the laboratory. The stage of estrous cycle was elucidated in virtue of the macroscopic findings of the ovaries and uterus (Sarkar et al., 2010). The CL was categorized into early luteal, mid luteal, late luteal and regressed stages with 10 CL at each stage. Further, luteal sample was frozen in liquid nitrogen and stored at -80^{o}C until RNA and protein extraction (Kumar et al., 2012).

2.2 Follicles collection

Ten follicles which seems healthy, transparent, highly vascular and with more than 14 mm diameter were utilized in this study. Preovulatory follicles were selected based on regressing CL and mucus secretion in uterus. Follicles were removed from ovaries and the theca externa were removed from the follicles with the help of forceps and stereomicroscope (Sarkar et al., 2010). The aspirated follicular fluid was stored at -20^{o}C for estimation of progesterone (P_4). Normal follicles are having relatively constant levels of P_4, so follicles having P_4 concentration less than 100 ng/mL were utilized in the present study (Kumar et al., 2012). Follicles were frozen with the help of liquid nitrogen and stored at -80^{o}C.

2.3 Determination of Hormone

As per the instruction of manufacturer (Immunotech, Czech Republic) using $P_4$125I RIA kit (IM1188) the P_4 concentration in FF were estimated. The FF was diluted with phosphate-buffered saline (PBS) with the dilution ratio of 1:5. 0.05 to 50 ng/mL was the measurable range. The inter and intra assay coefficients of variation was 7.2 and 6.5%, respectively.

Table 1 Target gene, primer sequences and amplicon length for qRT-PCR used in the study.

Gene	Sequence of nucleotide (5'-3')	Efficiency (%)	Amplicon length (bp)	EMBL accession No. or reference
BMP6	Forward:GGCCCCGTTAACTCGACTGTGACAA Reverse: TTGAGGACGCCGAACAAAACAGGA	101.2	108	XM_600972.2
RPS15A	Forward: AGGGCTGGGAAAATTGTTGTGAA Reverse: TGAGGGGATGGGAGCAGGTTAT	104.8	125	Mishra et al.,2015

BMP, Bone morphogenetic protein; **EMBL,** European molecular biology laboratory; RPS15A-Ribosomal protein 15A; qRT-PCR, Quantitative real time polymerise reaction.

2.4 Primers

Details of primers used are presented in Table 1. BMP6 primer was designed by employing Fast PCR software (6.5.63 version).

2.5 Quantitative RT-PCR

RNA was extracted from follicles and different stages of CL using TRIzol method (Invitrogen®). The integrity of RNA was assessed in 1% agarose gel with 1X tris-borate-EDTA (TBE) buffer, and purity and concentration of RNA was estimated in Nanodrop (Bio-Rad Laboratories, Hercules, CA). Constant amount of total RNA (1 µg) was reverse transcribed using cDNA Synthesis Kit (Thermo Fisher Scientific, Massachusetts, USA) and oligo-dT18 primer at 42^{o}C for 90 min. The resulting complementary DNAs were used in qPCR. The pre-ovulatory follicle (PF) was used as calibrator for obtaining relative mRNA expression.

The qPCR for each complementary DNA and the housekeeping gene RPS15A was performed in duplicate using SsoFast Eva GreenSupermix kit (Bio-Rad) in a Biorad CFX manager Real-Time qPCR System instrument as per manufacturers' instructions. The efficiency for the primer of different factors has been given in Table 1.

0.5 µL of cDNA as PCR template was added to 0.25 µL of forward and reverse primer (0.2 mM) each, and 5 µL of SsoFast Eva Green Supermix and final volume of 10 µL was adjusted with nuclease free water (NFW) and subjected to qPCR. The following general qPCR protocol was used for BMP6: enzyme activation for 30 sec at 95^{o}C, 40 cycles of a 3-segmented amplification and quantification program (denaturation for 5 sec at 95^{o}C, annealing for 10 sec at the primer-specific temperature (58^{o}C for BMP6, 60^{o}C for RPS15A) and elongation for 15 sec at 72^{o}C), a melting step by slow heating from 61 to 95°C with a rate of 0.58^{o}C/sec and continuous fluorescence measurement and a final cooling down to 4^{o}C. After the run ended, 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 (Biorad CFX manager Real-Time qPCR^{TM} software) qPCR efficiencies were determined by amplification of a standardized dilution series and slopes were obtained. The specificity of the product was checked in gel electrophoresis. Negative control was run by adding all the components except template to rule out the formation of primer dimer.

2.6 Antibodies

Immunoblotting and immunohistochemistry were performed using goat polyclonal GAPDH (sc-48166; Santa Cruz Biotechnology, Inc., Dallas, TX), rabbit polyclonal BMP6 (Catalog# YPA1178; Chongqing Biospes Co., Ltd), goat anti-rabbit IgG-HRP (sc-2004, catalogue no. D2114), mouse anti-goat IgG-HRP (sc-2354, catalogue no. B1815) and goat anti rabbit IgG-FITC (sc-2012, catalogue no. 1010).

2.7 Western blot

To obtain total proteins, liquid nitrogen triturated luteal tissues of different stages were suspended in RIPA lysis (Ameresco, UK) buffer and Halt protease inhibitor cocktail (Thermo Scientific,USA), homogenized, and centrifuged at 12,000g. Total protein concentration was estimated using Bradford protein assay and supernatant was diluted in sodium dodecyl sulfate (SDS) buffer (final concentration to 60 mM Tris, 2% SDS, pH 6.8, 10% glycerol and 100 mM dithiothreitol), followed by boiling for 5 min.

The protein samples (100 mg from each group of CL) were subjected to SDS 10% polyacrylamide gel electrophoresis, electro transferred onto polyvinylidene difluoride membrane, and blocked with 5% bovine serum albumin (BSA) before incubation with primary antibody namely, BMP6 at a 1:200 dilution and polyclonal GAPDH at a 1:500 dilution for overnight at 4^{o}C. After incubation, the membrane was washed thrice with PBS-T (PBS 0.01% Tween 20) for 5 min each and the respective secondary antibody conjugated with horseradish peroxidase was added and incubated at 37^{o}C for 1 h. After washing 3 to 4 times in PBS-Tween 20 solution, the positive signals were detected by incubating the membrane using 0.06% 3,30-diaminobenzidine tetrahydrochloride (Genei) in 1XPBS (pH 7.4) containing 0.06% H_2O_2 for 10 to 15 min. Under white light, the bands were visualized and captured with digital camera.

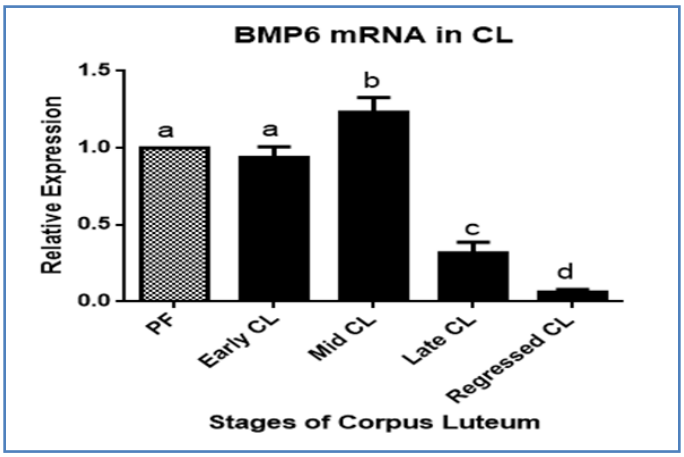

Figure 1 Expression of BMP6 at different stages of CL development in the buffalo (n = 10/group). (early CL, days 1–4, mid CL, days 5–10, late CL, days 11–16, and regressed CL, days >17 of estrous cycle). The PF served as calibrator group to calculate the fold change. RPS15A was used as reference gene to calculate ΔCt (delta C_t). One-way ANOVA was done to find the between group difference and Tukey honest significant difference test was done to find the pair-wise mean difference. Minimum level of significance was set at 95%. Each bar represents Mean ± SEM. Bars with different superscripts denote significant difference (P<0.05). Abbreviations: CL, Corpus luteum; mRNA, Messenger RNA; BMP, Bone morphogenetic protein; PF, Preovulatory follicle; RPS15A, Ribosomal protein 15A; ANOVA, Analysis of variance.

2.8 Immunohistochemistry

Freshly collected luteal tissues were fixed with 10% neutral buffer formalin, dehydrated through a series of graded alcohols, paraffin-embedded, serial-sectioned (5 µm), mounted on 3-Aminopropyl triethoxysilane (Thermo Scientific) coated slides and dried at 37°C overnight. Deparaffinization in xylene and rehydration in a series of graded alcohols at room temperature was done. Antigen retrieval was done in 10 mM sodium citrate buffer (pH 6.0, 0.05% Tween- 20) and rinsing thrice in PBS 5 min each. Blocking of non specific sites was done with 5% BSA for 2 h at 37°C. Subsequently, sections were probed with BMP6 antibody at 1:200 dilutions. Primary antibody was detected by fluorescent conjugated goat anti rabbit IgG-FITC secondary antibody at 1:400 dilution. Rinsing of slides were done thrice with the help of PBS and DAPI was used to stain nuclei. Without the addition of primary antibody, the control slides were stained with addition of isotype IgG. The slides were mounted with antifade solution (MP Biomedicals) and images were taken in AxioObserver.Z1 microscope (Germany).

2.9 Statistical analyses

All the experimental data were expressed as Mean ± SEM. The statistical significance of difference in mRNA expression of the examined factor across different stages of estrous cycle and the expression of protein was assessed using the software SPSS22 (IBM Corporation) by one-way analysis of variance followed by Tukey honest significant difference (HSD). Differences were considered significant if $P < 0.05$.

3 Results

3.1 Expression of mRNA for BMP6 in the corpus luteum

BMP6 was found to be expressed in a regulated manner with stage specific differences in the expression pattern in different stages of CL development in buffalo. The relative mRNA expression of BMP6 was significantly (P<0.05) upregulated during the mid luteal stage compared with the early and regressing stages of CL (Figure. 1). The expression of BMP6 in the early and late CL was comparable and significantly lower than the mid CL.

3.2. Western blot analysis

The BMP6 and GAPDH proteins were visualized on western blot analysis as bands of molecular weight approximately 54.4 kDa and approximately 37.5 kDa, respectively (Figure. 2A). The highest protein expression was identified during mid and late luteal stage which is correlated with mRNA expression.

3.3. Immunohistochemistry

The localization of BMP6 protein was conspicuous in various cell types in different stages of CL sections. The immunoreactivity was exclusively found in the cytoplasm of luteal cells and was greater during mid and late stages for BMP6. The negative controls did not show any specific immunoreactivity.

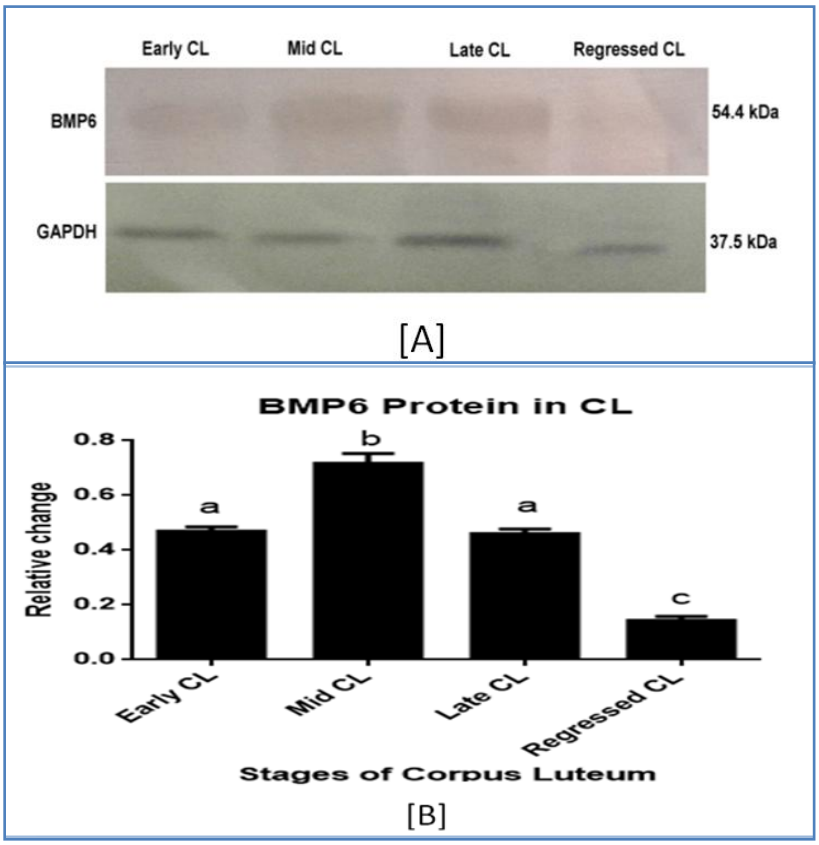

Figure 2 Demonstration of BMP6 protein by immunoblotting at different stages of CL development in the buffalo. Luteal protein was loaded at 100 mg/well, resolved in 12.5% SDS-PAGE and electrotransferred to the PVDF membrane. Primary antibody was used at 1:200 while secondary goat antirabbit antibody was used at 1:5000**.**

[A) Representative blot of BMP6 and GAPDH. The relative molecular weight of each BMP is indicated on the right end of each blot. GAPDH was used as reference protein **B)** Relative expression of BMP6 protein (Band Densitometric analysis of the immunoblot) was done using image J software (n=6/group). One-way ANOVA was done to find the between group difference and Tukeyhonest significant difference test was done to find the pair-wise mean difference. Minimum level of significance was set at 95%. Each bar represents Mean ± SEM. Bars with different superscripts denote significant difference ($P<0.05$). Abbreviations: BMP, Bone morphogenetic protein; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; CL, Corpus luteum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase ; PVDF, Polyvinylidene fluoride; ANOVA, Analysis of variance.]

4. Discussion

In the ovary, investigations on BMPs are mainly studied in the follicle of rat (Erickson & Shimasaki, 2003), cow (Glister et al., 2010) and ewe (Juengel et al., 2006). Except a solitary report by Erickson & Shimasaki (2003) on rat, no study could be found on BMPs in the CL. To the best of our knowledge, this is the first study on BMP6 in the CL of riverine buffalo. The mid CL showed a significantly increased expression of BMP6 transcripts (Figure. 1), which is consistent with the results of western blot (Figure. 2A and B) and immunofluorescence (Figure. 3).Though BMP6 mRNA was highly expressed in the theca cells and the oocytes of rat (Erickson & Shimasaki, 2003) and cow (Glister et al., 2004), it was only expressed in oocytes in the follicle of ewe (Juengel et al., 2006). In bovine GCs, BMP6 upregulated basal and IGF stimulated Estrogen (E_2) production (Glister et al., 2004). However, BMP6 suppressed P_4 production in the GC of rat (Otsuka et al., 2001a). In conclusion, BMP6 expression depends on the stage of CL development in the buffalo. Further studies are required to see its expression in the follicle and functional studies using follicular cell culture.

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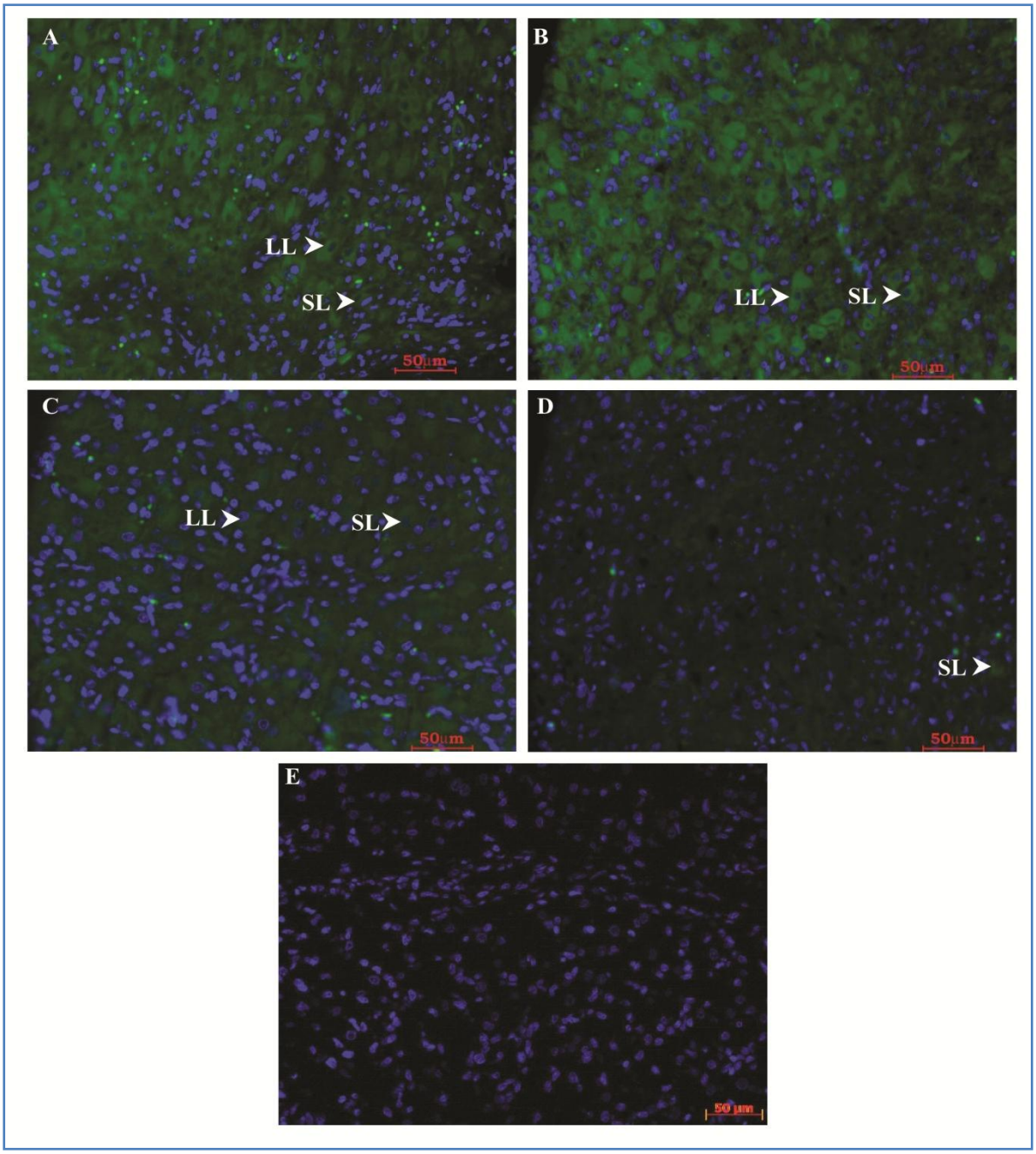

Figure 3 Fluorescent immunohistochemical localization of BMP6 in the CL of buffalo. Briefly, antigen retrieval was done using sodium citrate buffer method on 5 µm thick paraffin embedded sections of CL. BSA 5% was used to minimize the non-specific binding. Primary BMP6 antibody was used at 1:200 while the FITC was used at 1:400. Nucleus was counterstained with DAPI. Green filter was used while examination of the stained sections under the fluorescent microscope (Carl Zeiss Micro Imaging GmbH). Representative images from A through D indicate early, mid, late and regressed stages of CL. No primary antibody was used in the negative control (E). BMP6 was localized predominantly in the cytoplasm of large luteal cells of early and mid CL (A and B) while weak immunoreactivity was seen on late and regressed stages (C and D). Scale bar =50 µm. Abbreviation: LL, large luteal cell; SL, small luteal cell. BSA, Bovine serum albumin; FITC, Fluorescein isothiocyanate, DAPI, 4',6-diamidino-2- phenylindoledihydrochloride.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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