Transcriptional and translational abundance of Bone morphogenetic protein (BMP) 2, 4, 6, 7 and their receptors BMPR1A, 1B and BMPR2 in buffalo ovarian follicle and the role of BMP4 and BMP7 on estrogen production and survival of cultured granulosa cells


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ABSTRACT

BMPs and their receptors modulate the granulosa cell (GC) function in the follicle of domestic animals. Since little is known on BMPs in the buffalo, the present study was aimed to investigate the expression of BMP2, 4, 6, 7 and their receptors BMPR1A, BMPR1B, BMPR2 in the GC and theca cells (TC) of ovarian follicles and the role of BMP4 and BMP7 on GC. Follicles were classified into four groups based on size and E2 level in the follicular fluid as follows: (i) Group 1 (4–6 mm; < 0.5 ng/mL) (ii) Group 2 (7–9 mm; 0.5–5 ng/mL) (iii) Group 3 (10-13 mm; 5–40 ng/mL) and (iv) Group 4 (dominant follicle) (> 13 mm; > 180 ng/mL). The results revealed that except BMP6, BMP2, 4, 7 and receptors BMPR1A, BMPR1B and BMPR2 showed a minimum of 1.5–2 fold increase in mRNA expression in the GC of dominant follicle as compared to other follicle classes. In the dominant follicle, a two-fold increase in BMP4 and BMP7 expression was observed in the TC. At 100 ng/mL, the BMP4 and BMP7 either alone or in combination maximally down-regulated CASPASE3 and stimulated the transcripts of PCNA, FSHR and CYP19A1 that was supported by E2 secretion in the granulosa cell culture suggesting their role in cell survival and E2 production. In conclusion, GC and TC of dominant follicles express BMP 2, 4, 6, 7 and their receptors BMPR1A, BMPR1B and BMPR2. BMP4 and BMP7 stimulate E2 production and promote GC survival.

1. Introduction

Water buffalo is one of the most important dairy animals in the Asian countries. Indian buffaloes contribute to 57.8% of total population and India produces about 68% of the world's buffalo milk production (FAOSTAT, n.d.). The high milk fat content, lean meat and better conversion of roughage are the strengths of buffalo production. There is no religious taboo in the consumption of buffalo meat. Despite the merits, certain inherent reproductive problems like delayed puberty, silent heat, poor conception rate and long postpartum anoestrus period limit the lifetime productivity of the buffalo (Madan & Prakash, 2007; Perera, 2008).

Bone morphogenetic proteins (BMPs) are pleiotrophic growth factors that belong to the transforming growth factor-beta (TGF-β) super family. To date, over 20 BMPs are identified and shown to be involved in the regulation of cell proliferation, survival, differentiation and apoptosis, chondrogenesis, osteogenesis and embryogenesis. The BMPs have attracted much attention in the field of ovarian physiology. The expression of a range of BMPs within the different cell types of the antral follicle has been demonstrated in a variety of species including rodents, humans and ruminants (Elvin et al., 2000; Erickson & Shimasaki, 2003; Glister et al., 2004; Shimasaki et al., 2004). The granulosa cell-derived BMP2, BMP6 and the theca cell-derived BMP4 and BMP7 were found to promote granulosa cell (GC) proliferation, follicle survival and prevention of premature luteinization in the cow (Knight & Glister, 2006). The BMP receptor mRNAs are present in the ovary, with the strongest expression in GC and oocyte, which is consistent with the BMP actions observed on the GC in vitro (Shimasaki
<table>
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<tr>
<th>Gene</th>
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<th>Amplicon length (bp)</th>
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Abbreviations: BMP, Bone morphogenetic protein; FSHR, Follicle stimulating hormone receptor; CYP19A1, Cytochrome P450 family 19 subfamily A member 1; PCNA, Proliferating cell nuclear antigen; CASPASE3, Cysteine aspartic acid protease3; EMBL, European molecular biology laboratory.

The BMPs mediate the effects through activating the membrane bound serine/threonine kinase receptors (Xiao et al., 2007). The signal transduction of BMP is mainly mediated via the classical BMPs-Receptor-Smads signal pathway (Nohe et al., 2004). Binding of BMP ligands to the BMPR2 initiates the phosphorylation cascade. First, BMPR1A/B is phosphorylated, that subsequently phosphorylates receptor-activated Smad proteins (R-Smads), which associate with common mediator-Smad (co-Smad) and enter the nucleus, where they regulate gene expression. The Smad proteins regulate promoter activity by interacting with transcriptional co-activators or co-repressors to positively or negatively control gene expression (Xiao et al., 2007; Miyazono et al., 2005). A mitogen activated protein kinase mediated (MAPK-mediated) Smad independent pathway has also been reported to be involved in the BMP signaling (Cook & Evans, 2014).

The BMP4 and BMP7 regulate the process of follicle development through GCs in the cow (Shimizu et al., 2012a). The BMPs function as luteinization inhibitors by suppressing luteinizing hormone (LH) receptor expression in GC (Shimasaki et al., 2004). Additionally, the BMP system was shown to play a crucial role in folliculogenesis in human (Shi et al., 2009; Shi et al., 2010; Shi et al., 2011). Mounting evidence suggests that actively growing follicles that are destined for ovulation are the major targets of BMPs in the cow (Glister et al., 2004; Shimasaki et al., 1999). Given their role in ovarian follicular function, we hypothesized that BMPs and their receptor are expressed in the follicle and regulate the functions of GCs in the buffalo. Therefore, the present study was done with the following objectives: A) To evaluate the transcriptional, translational profile of BMP2, 4, 6, 7 and their receptors such as BMPR1A,1B and BMPR2 in the GC and TC of ovarian follicle during different stages of development in buffalo ovary; B) To study the effects of BMP4 and/or BMP7 on estradiol (E2) secretion, FSHR and CYP19A1 mRNA expression in the primary culture of GC and C) To study the effects of BMP4 and/or BMP7 on proliferating cell nuclear antigen (PCNA) and pro-apoptotic CASPASE3 mRNA expression in the primary culture of GC.

### 2. Materials and methods

All experimental protocols met the regulations of the Institutional Animal Care and Use Committee (IACUC).

#### 2.1. Collection of follicles and preparation

Twenty genitalia of normal, healthy, cyclic buffalo cows were collected at a local slaughter house within 10 to 20 min of exsanguination and were transported on ice to the laboratory. A total of forty ovaries were isolated from them. From each ovary, only healthy follicles were isolated followed by the isolation of GCs and theca cells (TC) of different stages as described earlier (Sarkar et al., 2010). The GC and TC isolated from each follicle were transferred into separate tubes and labeled. The GC in the flushing solution was centrifuged at 3000g for 10 min at 4 °C. The TC and GC pellet were separately snap frozen in liquid nitrogen and stored at -80 °C until RNA and protein isolation. The FF was stored at -20 °C until determination of progesterone (P4) and E2.

The FF were collected according to the E2 content (ng/mL) in the FF as follows: (i) Group 1 (FL1) < 0.5; (ii) Group 2 (FL2) 0.5–5; (iii) Group 3 (FL3) 5–40; and (iv) Group 4 (FL4) > 180 FF. The corresponding size of follicles were in the range of (i) 4–6 mm (FL1 or small); (ii) 7–9 mm (FL2 or medium); (iii) 10–13 mm (FL3 or large); (iv) >
Fig. 1. Left panel (A, C, E, G, I, K and M) represents the relative expression of BMP2, 4, 6, 7 and their receptors BMPR1A, BMPR1B and BMPR2 transcripts in the GC while the right one (B, D, F, H, J, L and N) indicates BMP2, 4, 6, 7 and their receptors BMPR1A, BMPR1B and BMPR2 transcripts in the TC of different follicular classes. Each bar represents Mean ± SEM. Bars with different superscript denotes significant difference P < 0.05). FL, Follicle; BMP, Bone morphogenetic protein; BMPR, Bone morphogenetic protein receptor; GC, Granulosa cell; TC, Theca cell.
13 mm (FL4 or/dominant/pre-ovulatory follicle) (Babitha et al., 2013). Forty ovaries, each with follicles, were used to extract 10 follicles/group for RNA extraction, immunoblotting and immunohistochemistry (IHC) studies.

### 2.3. Hormone estimation

Concentrations of P4 in the FF and E2 in FF and spent culture media of GC culture were estimated by P125I RIA kit (IM1188) and E2125I RIA kit, respectively (A21854) supplied by Immunotech, Czech Republic as per manufacturer's instruction. The measurable range was 0.05–50 ngmL\(^{-1}\) for P4 and 6–5000 pgmL\(^{-1}\) for E2. The FF was diluted at 1:100 dilutions with phosphate buffered saline (PBS). The intra- and inter-assay coefficients of variation were 6.5% and 7.2% for P4 and 12.1 and 11.2 for E2, respectively.

### 2.4. Primers

The primers used for qPCR in this study were designed using the Fast PCR (Version: 6.5.63) software. Details of the primers used are given in Table 1.

### 2.5. Quantitative qPCR analysis

Total RNA was isolated from GC and TC of all four follicular groups and cultured GCs by TRizol reagent (Invitrogen) according to manufacturer instructions. The RNA was treated with DNase 1 (Invitrogen) to remove any possible DNA contamination. Total RNA was quantified using nanodrop spectrophotometer (Eppendorf). The samples were adjudged pure when \(A_{260}/A_{280}\) O.D value was > 2.0. The integrity of total RNA was assessed by resolving in 1% agarose gel to visualize 18S and 28S subunits. Constant amounts of 1 \(\mu\)g of total RNA from FL (n = 10/group) were reverse transcribed using the RevertAid cDNA

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**Fig. 1.** (continued)
Synthesis Kit (Thermo scientific) and oligo-dT18 primer at 42 °C for 60 min. The resulting cDNAs were used in qPCR. The qPCR for each cDNA and the housekeeping genes beta actin and RPS15A was performed in duplicate using SsoFast Eva Green Supermix kit (Bio-Rad) in a Bio-Rad CFX manager Real-Time qPCR System instrument as per manufacturer’s instructions. In brief, PCR templates containing 0.5 μL reverse-transcribed total RNA were added to 0.25 μL forward primer (0.2 mM), 0.25 μL reverse primer (0.2 mM), and 5 μL of SsoFast Eva GreenSupermix to a final volume of 10 μL and were subjected to the general qPCR protocol for all investigated genes. The following general

Fig. 2. A) Representative western blots of BMP2, 4, 6 and 7 and their receptors BMPR1A, BMPR1B and BMPR2. B-H) Relative protein expression of BMP2, 4, 6, 7 and their receptors BMPR1A, BMPR1B and BMPR2. Each bar represents Mean ± SEM. Bars with different superscript denotes significant difference (P < 0.05). FL, Follicle; BMP, Bone morphogenetic protein; BMPR, Bone morphogenetic protein receptor.
qPCR protocol was used. Enzyme activation for 30s at 95 °C, 40 cycles of a 3-segmented amplification and quantification program (denaturation for 5 s at 95 °C, annealing for 10s at the primer-specific temperature (55 °C for PCNA, 56 °C for BMP7 and BMPR2, 58 °C for BMP2, BMPR1A and BMPR1B, 60 °C for BMP4, BMP6, FSHR, CYP19A1, CASPASE3, beta actin and RPS15A) and elongation for 15 s at 72 °C), a melting step by slow heating from 61 to 95 °C with a rate of 0.58°C/s and continuous fluorescence measurement, and a final cooling down to 4 °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 (Bio-Rad CFX manager Real-Time qPCR™ software). The cycle threshold (Ct) values were used to study relative expression. Beta actin and RPS15A were used as housekeeping genes and geometric mean of Ct values of beta actin and RPS15A was used as Ct of reference gene. The efficiency of the qPCR was determined by amplification of serially diluted standards, and slopes were obtained. The specificity of products was checked using analysis of melting temperature, and a high-resolution gel electrophoresis and further confirmed by sequence analysis. Negative control PCR containing all components except template was included for each sample to rule out the occurrence of primer dimer.

2.6. Antibodies and growth factors

Immunoblotting and immunohistochemistry were performed by using goat polyclonal GAPDH (sc-48,166; Santa Cruz Biotechnology, Inc., Dallas, TX), goat polyclonal BMP4 (sc-6896), rabbit polyclonal BMP2(YPA1176; Chongqing Biospes Co., Ltd), rabbit polyclonal BMP6 (YPA1178), rabbit polyclonal BMP7 (YPA1179), rabbit polyclonal BMPR1A (YPA1180), rabbit polyclonal BMPR1B (YPA1181), rabbit polyclonal BMPR2 (YPA1182), mouse anti-goat IgG-HRP (sc-2354), goat anti-rabbit IgG-HRP (sc-2004), goat anti rabbit IgG-FITC (sc-2012), bovine anti-goat IgG-CFL-647 (sc-362,284). Recombinant Human Bone Morphogenetic Protein 7 human recombinant, expressed in CHO cells (B1434) were procured for cell culture work.

2.7. Immunoblotting

Total proteins from the follicular tissues of different groups were isolated as described earlier (Babitha et al., 2014). The protein samples (100 μg from each follicle class) were resolved to 10–12.5% SDS-polyacrylamide gel electrophoresis, electro-transferred onto polyvinylidene difluoride (PVDF) membrane and blocked with 5% bovine serum albumin (BSA) before incubation with primary antibodies (@ 1:200 dilution) and polyclonal GAPDH (@ 1:5000 dilution) for overnight at 4 °C. After incubation, membrane was washed thrice with PBS-T (PBS + 0.01% Tween 20) for 5 min and subjected with respective secondary antibodies and incubated at 37 °C for 1 h. After washing 3 to 4 times in PBS-Tween 20, the positive signals were detected by incubating the membrane using 0.06% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Genei) in 1× PBS containing 0.06% H2O2 for 10–15 min. The bands were visualized under white light and recorded on a digital camera. Densitometry of the immune-specific bands was performed using Image J 1.43 U software (National Institute of Health, Bethesda, Maryland). The experiment was triplicated for each protein.

2.8. Immunohistochemistry

Immunolocalization of BMP2, BMP4, BMP6, BMP7 and their receptors BMPR1A, BMPR1B and BMPR2 were determined as described earlier (Babitha et al., 2014). Deparaffinization was carried out in xylene followed by rehydration in a series of graded alcohols at room temperature, epitope retrieval in sodium citrate buffer (10 mM sodium citrate, pH 6.0, 0.05% Tween-20), and blocked with 5% BSA for 2 h at 37 °C. Subsequently, sections were probed with BMPs and their receptors primary antibodies, at 1:200 dilutions. Primary antibodies were detected by fluorescent conjugated secondary antibodies at 1:400
dilution. The slides were rinsed and 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI @ 0.4 mg/mL) in PBS was applied to stain the nuclei of the cells in the follicle sections. The negative control slides were processed under similar conditions excluding primary antibody and addition of isotype IgG controls. Fluorescently stained sections were mounted with antifade media (MP Biomedicals) and images were captured using AxioObserver.Z1 (Carl Zeiss Micro Imaging GmbH, Germany) microscope.

2.9. Granulosa cell culture (GCC) and treatments

Ovaries were washed properly with physiological saline solution and GCs were collected from FL4 class (n = 4 follicles) and processed as described earlier (Babitha et al., 2014). The number of viable cells was determined using trypan blue exclusion. Finally, the processed GCs were re-suspended and plated out at 1.5 x 10^5 viable cells per well in a 24-well plate (total volume: 1 mL containing 10% FBS (Sigma) and Antibiotic & Antimycotic solution (Hyclone, Thermo Scientific) in a humidified CO2 (5%) incubator at 38.5°C. The cells were allowed to attach and grow (75%–80% confluent) for 48 h, and thereafter, the media was replaced with fresh media containing different concentrations (1, 10 and 100 ng/mL) of BMP 4 and/or BMP7 separately and were maintained over a period of 24, 48 and 72 h intervals. Control cells were grown in media without BMPs. After each incubation period the media were collected for E2 estimation and the cells were collected for mRNA isolation. Each treatment was tested in triplicate wells in each experiment.

Fig. 3. Fluorescent immunohistochemical localization of BMP2 in different follicular classes of riverine buffalo. Nucleus was counterstained with DAPI. Representative images A) FL1, B) FL2, C) FL3, D) FL4 and (E) negative control. Scale bar = 20 μm. FL, Follicle; GC, Granulosa cell; TI, Theca interna; TE, Theca externa; EC, Endothelial cell; FITC, Fluorescein isothiocyanate, DAPI, 4′, 6-diamidino-2- phenylindole dihydrochloride.
2.10. Statistical analyses

All experimental data are shown as Mean ± SEM. The statistical significance of difference in mRNA expression of the examined factors across different FL groups and the expression of protein was assessed using the software SPSS version.22 by one-way analysis of variance followed by Tukey’s honestly significant differences (HSD) and the E2 concentration and mRNA expression of FSHR, CYP19A1, PCNA and CASPASE3 in cultured GCs were assessed using the software SPSS.22 by two-way analysis of variance followed by Tukey’s honestly significant differences (HSD) as a multiple comparison test. The model included the main effects of the fixed factors (dose of BMPs) and their expression along the different time points (24, 48 and 72 h). Differences were considered significant if P < 0.05.

3. Results

3.1. Gene expression analysis

The FL1 class was used as calibrator for obtaining relative mRNA expression. RPS15A and beta actin were used as housekeeping gene. The intra-assay and inter-assay coefficient of variations were found to be below 5%. Efficiency corrected relative quantification of mRNA was obtained by Pfaff’s method (Pfaff, 2001). The relative fold change in the levels of BMP2 mRNA showed a significant increase in the GC of FL3 and FL4 as compared to GC of FL1 class (P = 0.0005; Fig. 1A) while it was significantly down-regulated in the TC of all class of follicles than FL1 (P = 0.0005; Fig. 1B). In contrast, the relative fold change in the levels of BMP4 mRNA in the TC was significantly greatest in FL4.

Fig. 4. Fluorescent immunohistochemical localization of BMP4 in different follicular classes of riverine buffalo. Nucleus was counterstained with DAPI. Representative images A) FL1, B) FL2, C) FL3, D) FL4 and (E) negative control. Scale = 20 μm. FL, Follicle; GC, Granulosa cell; TI, Theca interna; TE, Theca externa; EC, Endothelial cell; FITC, Fluorescein isothiocyanate, DAPI, 4′, 6-diamidino-2-phenylindole dihydrochloride.
The relative fold change in the levels of BMP4 mRNA was gradually increased from FL2 to FL4 and noted to be highest in GC of FL4 (Fig. 1C). The relative fold change in the levels of BMP6 mRNA in GC (P = 0.001; Fig. 1E) and TC (P = 0.041; Fig. 1F) followed a similar trend as BMP2 mRNA. The relative fold change in the levels of BMP7 mRNA was significantly up-regulated in the TC of FL4 (P = 0.0005; Fig. 1H) and did not modulate in the GC (P > 0.05; Fig. 1G) as compared to FL1. The relative fold change in the levels of BMPR1A (Fig. 1I), BMPR1B (Fig. 1J) and BMPR2 (Fig. 1M) mRNA revealed a significant increase in the GC of FL4 (P = 0.0005). In contrast, the relative fold change in the levels of BMPR1A (Fig. 1I), BMPR1B (Fig. 1J) and BMPR2 (Fig. 1N) mRNA were clearly downregulated in TC of all the FL classes as compared to control (P = 0.026). Interestingly, the GC of FL4 class showed a significant up-regulation of BMP2, BMP4, BMP6, BMPR1A, BMPR1B and BMPR2 mRNA except BMP7.

3.2. Immunoblotting analysis

The qualitative results of immunoblotting studies showed that the BMP2, 4, 6, 7 and their receptors BMPR1A, BMPR1B and BMPR2 were expressed in the GC and TC of follicle (Fig. 2A). Densitometric analysis revealed that BMP2 protein expression was significantly higher in the FL3 and FL4 (P = 0.001; Fig. 2B). Similarly, the BMP4 protein expression was significantly higher in all follicular classes as compared to FL1 (P = 0.037; Fig. 2C). In contrast, BMP6 protein expression was
feeble and did not differ across the different FL classes (P > 0.05; Fig. 2D). The BMP7 protein expression was found to be highest in FL2 than rest of the follicle classes (P = 0.003; Fig. 2E). The protein expression of BMPR1A (P = 0.0005; Fig. 2F), BMPR1B (P = 0.0005; Fig. 2G) and BMPR2 (P = 0.0005; Fig. 2H) showed a similar pattern and found to be greatest in FL4.

3.3. Immunohistochemistry of follicles

Immunofluorescence localization experiment revealed that the BMPs such as BMP2 (Fig. 3), 4 (Fig. 4), 6 (Fig. 5), 7 (Fig. 6) and the receptors BMPR1A (Fig. 7), BMPR1B (Fig. 8) and BMPR2 (Fig. 9) were localized in every class of follicle. In general, the FL3 and FL4 stages showed prominent expression as compared to FL1 while FL2 had moderate expression. However, the relative intensity of fluorescence showed a differential pattern between GC and TI in FL4. Theca interna of FL4 showed appreciable staining of BMP4 and BMP7 (Fig. 4D and 6D). In contrast, GC in FL4 showed intense expression of BMP2 (Fig. 3D). BMPR1A, BMPR1B and BMPR2 did not show a discernible differential expression in GC and TI. Interestingly, endothelial cells showed localization of BMP2 and 7 (Fig. 3D and 6B).
3.4. Effects of BMP4 and/or BMP7 on E2 and its synthetic pathway mRNA expression in GCC

Stimulation of the primary GCC derived from the FL4 class of buffalo with increasing concentrations of BMP4 revealed a highly significant increase in the accumulation of E2 over a period of 72 h (P = 0.0005; Fig. 10C) except control. BMP4 @ 100 ng/mL showed a highly significant up-regulation FSHR (P = 0.0005; Fig. 10A) and CYP19A1 (P = 0.0005; Fig. 10B) mRNA expression as compared to other doses except at 24 h of culture. Effect of BMP7 showed a highly significant time effect over a period of 72 h for E2 (P = 0.0005; Fig. 11C) except control. At 100 ng/mL, BMP7 showed the highest values for E2 (P = 0.0005; Fig. 11C) at 72 h that corresponded well with the relative fold change of transcripts such as FSHR (P = 0.0005; Fig. 11A) and CYP19A1 (P = 0.0005; Fig. 11B). The combined effect of BMP4 and BMP7 was tested at 100 ng/mL which revealed a highly significant increase in the FSHR (P = 0.0005; Fig. 12A) and CYP19A1 (P = 0.0005; Fig. 12B) at 72 h. However, E2 (Fig. 12C) production at 72 h failed to show significant difference between BMP7 and combined effect. The results revealed that the combined effect was significant but not additive.

3.5. Effects of BMP4 and/or BMP7 on apoptotic pathway markers PCNA and CASPASE3 mRNA expression in GCC

The PCNA transcripts showed a significant increase with respect to time and concentration for BMP4 as well as BMP7 (P < 0.05). The interaction was significant at 72 h for 100 ng/mL as compared to other
concentrations ($P = 0.0005$; Fig. 13A and C). Though the combined effect of BMP4 and BMP7 showed significance at 72 h, the increase was less than additive ($P = 0.0005$; Fig. 13E). In contrast, the relative change in the apoptotic CASPASE3 showed a significant decrease at concentration 100 ng/mL for BMP4 and BMP7 at 72 h ($P = 0.0005$). However, the combined effect of BMP4 and BMP7 showed a non-significant decrease in CASPASE3 at 72 h. Overall, the combination experiment showed that BMP7 at 100 ng/mL showed a significant increase of PCNA and decrease of CASPASE3 at 72 h in the GCC of buffalo.

4. Discussion

The expression of BMPs and their receptors in the ovarian follicle was first described in the rat (Shimasaki et al., 1999). Subsequently, different workers reported its presence in different species like cow (Glister et al., 2004; Fatehi et al., 2005; Glister et al., 2010), ewe (Souza et al., 2002; Juengel et al., 2006), and human (Shi et al., 2009; Shi et al., 2010; Shi et al., 2011; Akiyama et al., 2014). Apart from the follicle, transcript abundance studies have shown the presence of BMPs in the oviduct of cow (Garcia et al., 2014). The present results assume significance as little is known on the expression and probable function of BMP system in the bubaline follicle. There was a reasonable...
congruence among the qPCR, immunoblotting and immunohistochemistry studies on the expression profile of BMPs such as BMP2, 4, 6, 7 and the receptors BMPR1A, BMPR1B and BMPR2 (Figs. 1 to 9). One of the most consistent finding of the present study is that FL4 class, which represents the actively growing follicle destined for ovulation, showed significant up-regulation of BMPs such as BMP2, 4, 6, 7 and the receptors BMPR1A, BMPR1B and BMPR2 as compared to FL1. The result is in consistent with the findings of Glister et al. (2010) (Glister et al., 2010) who reported abundance of BMP and their receptors in the estrogenically active large follicle of cow as compared to atretic one. Similarly, GC of pre-ovulatory follicles showed a significant increase in the transcripts of BMP2, BMPR1A, and BMPR1B in the bovine (Selvaraju et al., 2013). However, a differential expression was seen between GC and TC of FL4 for different BMPs. For instance, GC showed a significant up-regulation of BMP2, 4, 6, 7 and the receptors BMPR1A, BMPR1B and BMPR2, while TC showed an up-regulation of BMP4 and BMP7 and down-regulation of the receptors such as BMPR1A, BMPR1B and BMPR2. This observation is in agreement with the previous studies in the rat and cow where it is shown that BMP4 and BMP7 are preferentially expressed in the TC (Erickson & Shimasaki, 2003; Glister et al., 2010). Transcript abundance and/or immunohistochemical studies showed up-regulation of BMP2 in the GC of

**Fig. 9.** Fluorescent immunohistochemical localization of BMPR2 in different follicular classes of riverine buffalo. Nucleus was counterstained with DAPI. Representative images A) FL1, B) FL2, C) FL3, D) FL4 and (E) negative control. Scale bar = 20 μm. FL, Follicle; GC, Granulosa cell; TI, Theca interna; TE, Theca externa; EC, Endothelial cell; FITC, Fluorescein isothiocyanate, DAPI, 4′, 6-diamidino-2-phenylindole dihydrochloride.
antral follicle in the cow (Kayani et al., 2009) and ewe (Juengel et al., 2006). In contrast, Fatehi et al. (2005) reported up-regulation of BMP2 in the TC. Up-regulation of BMPR1A, BMPR1B and BMPR2 in the GC and its down-regulation in the TC observed in our study is line with the results in the cow (Glister et al., 2010). Though BMP6 was present in the GC and TC of different classes of follicle, the expression was variable. Immunocytochemical studies showed localization of BMP6 to GC in the cow (Glister et al., 2004). In contrast, the same group reported up-regulation of BMP6 transcripts in the TC (Glister et al., 2010). Type and activity of the follicle, differential sensitivity of the experimental techniques and species specific difference are the probable reasons behind the conflicting reports.

The BMP4 and BMP7 were selected for the in vitro studies as they are widely used to study the regulation of steroid production in the follicular GC of different species (Akiyama et al., 2014; Lee et al., 2001; Kayamori et al., 2009; Shimizu et al., 2012b). The concentration chosen was based on the reports in the human (Akiyama et al., 2014) and cow (Shimizu et al., 2012b). Follicles of FL4 class were used to generate primary culture, as it is an established methodology in our lab (Gupta et al., 2015). Our results show that at 72 h of GCC, 100 ng/mL of BMP4 and BMP7 stimulated the transcripts of FSHR and CYP19A1 (aromatase) significantly that correlated well with E2 accumulation in the culture media (Figs. 10 and 11). BMP7 was potent in terms of expression of FSHR, aromatase and E2 at 100 ngmL−1 as compared to BMP4. An increase in the FSHR observed in the present study is in consistent with the observation that BMP7 and BMP 6 @ 100 ngmL−1 stimulated the expression of FSHR in the GC of human (Shi et al., 2010). The same group reported up-regulation of FSHR and CYP19A1 in response to treatment with BMP2. It is reported that BMP4 and BMP7 increased FSH induced E2 in the GC of rat (Shimasaki et al., 1999) but not in the cow (Glister et al., 2002). However, basal and IGF induced secretion of E2 and inhibin was enhanced by BMP 4 and BMP7 in the GC of cow (Glister et al., 2004; Shimizu et al., 2012b) and rat (Shimasaki et al., 1999; Selvaraju et al., 2013). Treatment of GCC with BMPs in the presence of FSH decreased the P4 production and the related enzymes in the rat (Shimasaki et al., 1999; Selvaraju et al., 2013) and ewe (Pierre et al., 2005). Combined effects of BMP4 and BMP7 did not show advantage over the individual effect. Taken together, the previous reports support our observation that treatment of GCC with BMP4 or BMP7 increase E2 production.

Higher concentrations of BMP4 or BMP7 significantly increased the transcripts of PCNA and decreased the levels of transcripts of CASPASE3 at 72 h in the GCC (Figs. 12 and 13). Our findings in this regard support the studies by Shimizu et al. (2012a, 2012b) who reported a significant decrease in the apoptosis of bovine GC following treatment with BMP4 and BMP7. Similarly, BMP4, BMP6 and BMP7 increased the proliferation of GC in the cow (Glister et al., 2004). In contrast, in the ewe, BMP4 or BMP7 failed to stimulate the proliferation of GC (Juengel et al., 2006).

Collectively, our results show that BMPs (BMP2, 4, 6 and 7) and their receptors (BMPR1A, BMPR1B and BMPR2) are present in the GC and TI of follicles in the buffalo with prominent expression in the FL4 class. In addition, BMP4 and BMP7 stimulate estradiol production and GC survival in buffalo granulosa cells.
Fig. 11. The mRNA expression of FSHR (A), CYP19A1 (B), and E2 production in cultured GCs treated with BMP7 at dose rate (0, 1, 10, 100 ng mL$^{-1}$) for 24 h, 48 h and 72 h incubation period. Only F4 GCs (n = 4) were taken for study. All values are shown as mean ± SEM. Different superscripts denote statistically different values (P < 0.05). BMP7, Bone morphogenetic protein 7; FSHR, Follicle stimulating hormone receptor; CYP19A1, Cytochrome P45019A1; E$_2$, Estradiol.
Fig. 12. The mRNA expression of FSHR (A), CYP19A1 (B), and E2 production in cultured GCs treated with BMP4 (100 ngmL<sup>-1</sup>), BMP7 (100 ngmL<sup>-1</sup>) and a combination of BMP4 and BMP7 (each at 100 ngmL<sup>-1</sup>) for 24 h, 48 h and 72 h incubation period. Only F4 GCs (n = 4) were taken for study. All values are shown as mean ± SEM. Different superscripts denote statistically different values (P < 0.05). BMP7, Bone morphogenetic protein 7; FSHR, Follicle stimulating hormone receptor; CYP19A1, Cytochrome P45019A1; E<sub>2</sub>, Estradiol.
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