EXPRESSON PROFILE OF GROWTH DIFFERENTIATION FACTOR 9 (GDF-9) AND BONE MORPHOGENETIC PROTEIN 15 (BMP-15) GENES IN BUFFALO OOCYTES

K. Muralidharan¹, S. Eswari², K. Vijayarani³

ABSTRACT

Growth differentiation factor 9 (GDF-9) and its closest homologue bone morphogenetic protein 15 (BMP-15), the oocyte specific paralogs of the TGF-α super family, play synergistic roles during follicular development in mammalian ovary, with momentous impact on overall female fertility and prolificacy. There is little knowledge about the expression pattern of these two genes in buffalo. This paper depicts the temporal expression pattern of GDF-9 and BMP-15 mRNA in cumulus free oocytes (CFOs), cumulus cells (CCs), and granulosa cells (GCs) retrieved from the follicles of buffalo ovary, by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The total RNA was isolated from pooled samples of cumulus free oocytes (CFOs), cumulus cells (CCs) and granulosa cells (GCs) of cumulative oocyte complexes (COCs) of 2064 buffalo ovaries, collected from the local abattoir, using RNeasy Micro Kit (Qiagen, USA), were used in this study. The mRNA transcripts encoding GDF-9 and BMP-15 were detected by RT-PCR using specific oligonucleotide primers. PCR product of an expected 401 bp for GDF-9 and 377 bp of BMP-15 were obtained in CFOs, CCs and GCs. It is concluded that the present study affirmed oocyte specific mRNA expression of GDF-9 and BMP-15 in buffalo ovary, besides establishing unique expression in other cells, such as CCs and GCs of COC as well.

KEY WORDS

Buffalo, BMP-15, Cumulative Oocyte Complex, GDF-9, mRNA

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INTRODUCTION

The potential of an oocyte to develop into viable embryo depends on the accumulation of RNA or imprinted genes during the process of oogenesis. Thus, there is general agreement that the developmental competence of oocyte could be related to the abundance of specific mRNA transcript pool that is accumulated during oocyte growth and at final phases of folliculogenesis (Sirard et al., 2006). However, there is limited information about the control of gene transcription in the oocyte.

Members of the transforming growth factor-beta (TGF- \( \beta \)) super family are known to be important regulators of proliferation and differentiation of several types of cells. Growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) are oocyte secreted paralogs of the TGF- \( \beta \) super family, co-expressed in the oocyte during follicular development, and have been shown to have much greater biological synergistic activity compared to individual action (Su et al., 2004).

The expressions of GDF-9 and BMP-15 have been reported in the oocytes of cows (Bodensteiner et al., 1999; Hosoe et al., 2011), sheep (Bodensteiner et al., 1999; McNatty et al., 2005), and goats (Silva et al., 2005). However, there is meager information on the expression pattern of GDF-9 and BMP-15 in buffalo oocytes. The present study aimed to explore the expression profile of GDF-9 and BMP-15, the novel members of TGF- \( \beta \) super family from buffalo ovarian follicles.

MATERIALS AND METHODS

**Oocyte recovery and grading:** The buffalo ovaries (n=2064) collected from the local abattoir and brought to the laboratory within 2-3 hours of slaughter, were washed five times in modified Dulbecco’s phosphate buffered saline (mDPBS) containing 100 IU/ml penicillin and 100 µg/ml streptomycin. The cumulus-oocyte complexes (COCs) were recovered by aspiration of non-atretic antral follicles (3-8 mm diameter) in oocyte collection medium (OCM), and were pooled in a sterile 50 ml conical bottom centrifuge tube.

The tubes were kept at 37 °C for 15 minutes for the settlement of COCs. The sediment was mixed with 2 ml of OCM and screened for COCs under stereozoom microscope (Nikon, Japan). The aspirated COCs were graded on the basis of morphological appearance of the cumulus cell investments and homogeneity of ooplasm as described by Nandi et al. (1998). Qualitatively good (Grade A and Grade B) oocytes were deliberated for expression analysis.

**Collection of samples for total RNA isolation:** The selected COCs were treated with 0.1 per cent trypsin-EDTA solution for 2-3 minutes to denude the surrounding cumulus cells and washed 2-3 times in mDPBS. The cumulus free oocytes (CFOs) were observed under stereo zoom microscope, to ensure that they were free of cumulus cells.

The CFOs were transferred to a 1.5 ml capacity micro-centrifuge tube with minimum amount of PBS, and then frozen at -80 °C before proceeding for
RNA extraction. The remaining cumulus cells were transferred to a 1.5 ml capacity centrifuge tube and centrifuged at 300 g for 2 minutes. The supernatant was discarded and the cell pellet was resuspended with minimum amount of PBS and stored at -80°C for RNA isolation.

The granulosa cells were collected from the ovaries following the method described by Edwards et al. (2008). After the removal of COCs from follicular fluid, the follicular debris was removed from the granulosa cells using a glass pipette and examined under stereo zoom microscope. The granulosa cells (approximately 1.5-2.0 x 10⁶) were transferred into a 1.5 ml tube and centrifuged for 5 minutes at 300 g in a table top micro-centrifuge.

The cell pellets were washed twice in PBS by centrifugation at 300 g for 5 minutes. The supernatant was discarded, and the cells were stored at -80°C with minimum volume of PBS for RNA extraction.

**Total RNA isolation:** Total RNA was isolated from the pooled samples of cumulus free oocytes (CFOs), cumulus cells (CCs) and granulosa cells (GCs) using RNeasy Micro Kit (Qiagen, USA) according to the manufacturer’s instructions.

**Complementary DNA synthesis:** The cDNA synthesis was carried out using Revert Aid M-MuLV Reverse Transcriptase Kit (Fermantas, USA) in a standard 20 μl reaction mixture. One microgram of total RNA was used as template RNA for reverse transcription as per manufacturer’s instructions in the kit protocol.

**Polymerase chain reaction:** The synthesized first strand cDNA was used for amplification by polymerase chain reaction (PCR) using gene specific primers. Specific primers for GDF-9 and BMP-15 were used for PCR reaction (Table-1). Bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control for PCR. The programme comprised an initial denaturation step at 95°C for 5 minutes, followed by denaturation at 95°C for 30 seconds, annealing of GDF-9 (52°C) and BMP-15 (56°C) for 30 seconds, and extension at 72°C for 1 minute and final extension at 72°C for 5 minutes. Each PCR was performed for 30 cycles for each sample. The number of cycles was optimized for each set of samples by a gradient method over the range of 20-40 cycles to ensure that amplification of cDNA for all primer sets was terminated in the exponential phase of the PCR.

**Confirmation of RT-PCR amplicons:** Products of the RT-PCR were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide (EtBr) staining. The 1.5% agarose gel prepared in TAE buffer was melted and casted on a gel tray after adding 1 μl of EtBr as intercalating dye. After solidification, the gel was placed into the electrophoresis tank containing 1X TAE buffer. Then, 12.5 μl of each amplified product and 5 μl of 100 bp ladder along with GAPDH gene (positive control) were electrophoresed. The results obtained were recorded in a Gel documentation system (GELDOC, USA).
Table-1. PCR Primers* used for detection of GDF-9 and BMP-15 expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'→ 3')</th>
<th>Size (bp)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDF-9 (Forward)</td>
<td>AGAAGCTGCTGAGGTGTAAGATT</td>
<td>401</td>
<td>AB058416</td>
</tr>
<tr>
<td>GDF-9 (Reverse)</td>
<td>AAGCAATGAGCCATCGGC</td>
<td>401</td>
<td>AB058416</td>
</tr>
<tr>
<td>BMP-15 (Forward)</td>
<td>CAAACGGACGATTCGATCTGAA</td>
<td>377</td>
<td>AY572412</td>
</tr>
<tr>
<td>BMP-15 (Reverse)</td>
<td>TCAACCTACATGTCAGGACTGG</td>
<td>377</td>
<td>AY572412</td>
</tr>
</tbody>
</table>

*Hosoe et al. (2011)

RESULTS AND DISCUSSION

The expression pattern of mRNA transcripts of GDF-9 and BMP-15 genes, studied in CFOs, CCs and GCs in buffalo follicles by RT-PCR, and the resultant product amplicons electrophoresed, are presented in Figure-1 (a-c). A single 497 bp product for GAPDH is obtained in all the RNA samples used in the study, indicating that there was no contamination by genomic DNA during RNA extraction. PCR product of an expected 401 bp for GDF-9 and 377 bp product of BMP-15 were recorded in CFOs, CCs and GCs. The identities of PCR products were further confirmed by sequencing and the homology was tested with the sequences of other species (DNA STAR).

GDF-9 is predominantly expressed in the ovary and the same is true for its closest homologue, BMP-15, which exhibits gene and protein expression patterns closely resembling those for GDF-9 (Jaatinen et al., 1999). GDF-9 in cows and sheep has been found to be selectively expressed in oocytes from primordial follicles (Bodensteiner et al., 1999). On the other hand, BMP-15 is expressed in the oocyte from the primary follicle stage continuing through ovulation (Laitinen et al., 1998). In the present study also, GDF-9 and BMP-15 were expressed in oocytes of follicles of buffalo ovary, as observed in earlier ovarian follicular expression studies in sheep (McNatty et al., 2005) and goats (Silva et al., 2005).

The cumulus cells transmit molecules through gap junctions that are required to support oocyte growth, development and regulate meiosis and transcriptional activity in the oocyte genome (Tanghe et al., 2002). Recent studies in human (Margoulis et al., 2009) have suggested the presence of BMP-15 and GDF-9 in granulosa and/or cumulus cells as well as in oocytes of large antral follicles, which might regulate meiosis and extrusion of polar body.

The present study revealed the expression of GDF-9 and BMP-15 in cumulus and granulosa cells. Similar expression patterns have been reported in other species, viz., human (Asou et al., 2006), goat (Silva et al., 2005) and cows (Hosoe et al., 2011). On the contrary, Sendai et al. (2001) has reported GDF-9 mRNA expression in oocytes, but not in cumulus and granulosa cells in bovine. Duffy (2003) reported that granulosa cells adjacent to the oocyte as well as the oocytes have been shown to express GDF-9 mRNA and protein in primates. Margulis et al. (2009) reported that BMP-
15 was expressed not only in oocytes, but also in human granulosa cells.

The expression patterns of GDF-9 and BMP-15 in the pre-ovulatory and growing follicles suggest that they could be playing paracrine role in the regulation of differentiation and proliferation of granulosa cells. Moreover, oocyte specific expressions of GDF-9 and BMP-15 within the ovary could be related to their role in follicular and oocyte developments in an autocrine manner.

Figure-1. Agarose gel (1.5 %) showing PCR amplicons of GDF-9 (401 bp), BMP-15 (377 bp) and GAPDH (497 bp) from cumulus free oocytes, (a) cumulus cells, (b) granulosa cells, (c) Lane 1: 100bp ladder, Lane 2: GDF-9, Lane 3: BMP-15 and Lane 4: GAPDH.

CONCLUSION

The presence of members of TGF-β super family, e.g., GDF-9 and BMP-15 might be indicative of their role on a wide variety of growth and differentiation processes in a number of tissues within the buffalo ovary. The oocyte specificity of GDF-9 and BMP-15 mRNA expression is consistent with the concept that the oocyte secreted growth factors might be playing a role in the regulation of follicular development. Our study indicated intra-follicular granulosa cell mRNA expression of the above genes from buffalo ovarian follicles. It supports the concept that early stages of ovarian follicular growth and development are regulated by intra-ovarian factors. To move forward with these findings, the quantitative level of differential expression dynamics of GDF-9 and BMP-15 genes from buffalo ovary needs further research.

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REFERENCES


Duffy, D.M. 2003. Growth differentiation factor-9 (GDF-9) is expressed by the primate follicle throughout the periovulatory


