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Expression Profile and Characterization of Genes Involved in Bidirectional Communication during in Vitro Maturation of Buffalo (Bubalus Bubalis) Follicular Oocytes

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Abstract—*Present study was undertaken to evaluate the spatial and temporal expression of GDF-9, BMP-15, HAS-2 and Cx-43 genes during in vitro maturation of Buffalo follicular oocytes and cumulus cells. Above said genes play major role in oocyte and cumulus cross talk during folliculogenesis and in vitro maturation of oocytes which ultimately affects their developmental competence. A quantitative RT-PCR system was standardized to measure transcript abundance of selected genes at different time intervals of 0,8,16 and 24 Hr in the presence of Gonadotropins. Their spatial expression was also determined. GDF-9 and BMP-15 both were found to be expressed only in the oocytes whereas Cx-43 mRNA expressed only in cumulus cells. Buffalo oocytes and cumulus cells both expressed HAS-2 mRNA. Analysis of temporal expression of these genes revealed that both GDF-9 and BMP-15 were upregulated with the progression of IVM period ($p<0.05$), however; decrease in expression of all mRNA transcripts except HAS-2 was evident after 24 h of in vitro maturation. It could be interpreted that GDF-9 and BMP-15 work in synergism to influence HAS-2 during IVM under the influence of FSH and LH. The genes under study were also characterized in terms of their nucleotide sequence.*

Complete cDNA sequence of GDF-9 (1378 bp), BMP-15 (1185 bp) was deciphered and submitted in NCBI GenBank with Accession Nos. EF202171, EF375880 respectively. Analysis of these sequences revealed nucleotide and amino acid variations at several places with respect to bovine sequences. Both GDF-9 and BMP-15 were found to have conserved six cysteine residues and N-glycosylation sites.

Keywords*: In vitro maturation, oocyte, GDF9 expression, bidirectional communication.*

1. INTRODUCTION

Various endocrine, autocrine, paracrine factors and gap junctional proteins mediate cellular communication between oocytes, cumulus cells and the mural granulosa cells [1]. Differential expression of genes involved in bidirectional communication, during in vitro maturation may affect the developmental competence of oocytes [2]. Genes expressed in cumulus cells influence not only the quality of oocytes and cumulus cell functions but also the subsequent embryonic development and implantation potential of resulting embryos [3]. GDF-9 and BMP-15 both have been shown to promote cumulus expansion *in vitro*, and also upregulate the expression of hyaluronan synthetase (HAS-2) which is required for the synthesis of hyaluronan in the extracellular matrix [4]. The interconnection between the cumulus cells and the oocytes and between the cumulus cells themselves are maintained by gap junctions called as connexins e.g. Cx-37 and Cx-43 which are expressed by oocyte, cumulus, granulosa, theca cells and luteal cells of equines, bovines and humans [5].

Buffaloes (Bubalus bubalis) are considered as the primary dairy animal is India. The IVF success rate in buffalo is reported to be not more than 10-15% [6]. In this regard, study of effect of Gonadotropins which are main components of in vitro maturation media on the genes responsible for bidirectional communication between the oocytes and surrounding cumulus cells may lead to important information in defining the oocyte competence. In the light of above facts, present work was undertaken to study the effect of FSH and LH on expression level of GDF-9, BMP-15, HAS-2, and Cx-43 genes during IVM to interpret their role in the bidirectional communication process.

2. MATERIALS AND METHODS

2.1 Collection of ovaries and isolation of COCs

COCs were collected by aspiration from visible follicles on the surface in oocyte collection medium . Oocytes with homogeneous cytoplasm and having at least 4/5 layers of cumulus cells were selected for *in vitro* maturation and subsequent studies.

2.2 IVM of COCs and experimental groups

Pools of 20 randomly selected COCs were cultured in 50µl maturation drops and maintained at 38.5^0C in 5%CO_2 in air

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atmosphere. The control *in vitro* maturation medium was supplemented with any one of the two supplements under study. Treatment groups: 1. Control medium 2. Control medium with 5 µg/ml pFSH (Sigma) 3. Control medium with 10 µg/ml LH (Sigma). COCs were taken out from maturation drops at the intervals of 0 (i.e. just after collection), 8, 16 and 24 hours after assessing the cumulus expansion. They were then rinsed in PBS, frozen dry in liquid nitrogen, and kept at -80^{0}C. For further analysis, only oocytes that reached metaphase II after in vitro culture and associated cumulus cells were used.

2.3 RNA extraction and RT-PCR

Total RNA from oocytes and cumulus cells was extracted with Cell to cDNA II kit (Ambion) with some modifications.

2.4 Selection of primers

Primers were designed using the web-based software PRIMER-3. For GDF-9, upstream primer and the downstream predicted a 497 bp product. Primers for BMP-15 predicted a 498 bp product. Primers for HAS-2 predicted a 280 bp product. Cx-43 primers used in the present study have been reported by Rizos et al. [7] and predicted a 293 bp product. The primers for 18S predicted an amplification product of 337 bp.

2.5 Amplification of GDF-9, BMP-15, HAS-2 and Cx-43 cDNA by PCR

PCR reactions were set in 25 µl mixture containing 1X PCR buffer, 0.4 µM of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 1.25U Taq DNA Polymerase (Promega).

Each PCR amplification consisted of an initial denaturing reaction (94^{0}C, 4 minutes), annealing (GDF-9 60^{0}C/30S; BMP-15 54^{0}C/30S; HAS-2 54^{0}C/30S; Cx-43 55^{0}C/30S and 18S rRNA 62^{0}C/30S) and extension of 72^{0}C/30S for GDF-9, BMP-15, HAS-2, and Cx-43 and 72^{0}C/28S for 18S rRNA. Densitometry data for band intensities was generated using AlphaDigiDoc™ AD-1201 software under Windows™ environment. To quantify specific gene expression in COCs, the levels of expression of specific oocyte and granulosa cell mRNAs in each treatment were calculated relative to 18S rRNA to normalize the experimental variations.

2.6 Transcript Characterization

To characterize the nucleotide sequence of GDF-9 and BMP-15, the cDNA sequence of the respective gene was divided into overlapping fragments. GDF-9 mRNA sequence was divided in three overlapping fragments of size 731, 675 and 497 bp, which were sequenced and analyzed. To amplify BMP-15, two overlapping fragments of size 844 and 497 bp were amplified and sequenced.

2.7 Statistical Analysis

Relative abundance data for each transcript was expressed relative to 18S rRNA amplification value under the same experimental group. Data obtained from different experiments, was analyzed by software Stata version 9 using ANOVA for comparing expression level of different genes at different hour intervals.

3. RESULT AND DISCUSSION

3.1 Optimization of PCR exponential phase

32 cycles of PCR were optimized as the exponential phase for GDF-9, BMP-15 and HAS-2. For 18s rRNA 24 cycles were found to be at exponential phase through initial experiments.

3.2 Site of expression genes under study

GDF-9 and BMP15 were found to be expressed in oocytes only and not in the cumulus cells (Fig. 1A,B). Thus GDF-9 and BMP-15 are expressed in oocytes of Buffalo. However we found with buffalo oocytes, HAS-2 expressed both in oocytes and cumulus cells (Fig. 1C). Cx-43 mRNA has been reported to be expressed in cumulus cells, although some reports are available confirming its expression in oocytes also, in rat and bovine [8]. In the present study, we found amplification of Cx-43 in cumulus cells not in oocytes (Fig 1D).

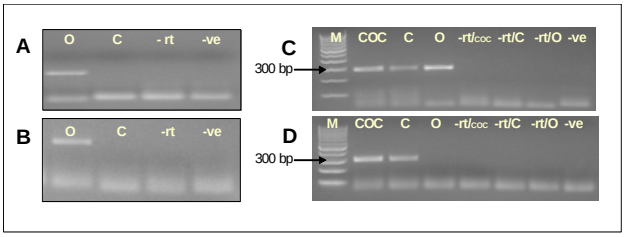

Fig. 1: Gel photograph showing the site of expression of A.GDF-9 B. BMP-15 C. HAS-2 and D. Cx-43. O: oocyte, C: Cumulus Cells, COC: Intact cumulus oocyte complexes, -rt: RT negative controls from oocyte/ cumulus/ COC cDNA preparations, -ve: PCR negative controls.

3.3 Expression of GDF-9, BMP-15, HAS-2 and Cx-43 at different hour intervals during IVM

Groups supplemented with FSH and LH showed significant increase in expression of GDF-9 upto 16 h and then expression decreased after 24 h of IVM. Like GDF-9, BMP-15 RA was least at 0 h of maturation (as compared to experimental groups at other hour intervals) which steadily increased up to 16 h and then reduced significantly after 24 h of in vitro maturation.

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Fig. 2 Expression of GDF-9 and BMP-15

Expression of HAS-2 was found to be up regulated till 24 h, maximum expression was observed at 16 h and then nonsignificant decrease was observed after 24 h. However, HAS-2 expression at 24 h was more as compared to 0 h expression. This trend of expression was same in both experimental groups.

Fig. 3 Expression of HAS2 and Cx-43

In all experimental groups as well as in control groups the expression pattern revealed upward trend from 0-6 h and further upto 16 h (non significantly) followed by a downtrend upto 24 h.

3.7 Nucleotide and Amino acid sequence of buffalo GDF-9 (Accession no. EF202171)

Buffalo GDF-9 cDNA consist of an open reading frame of 1362bp coding for 453 amino acid residues of 51.89 Kd molecular weight. A multiple sequence alignment of buffalo GDF-9 cDNA sequence was carried out with bovine GDF-9 sequence available in the GenBank (AB058416). A total of nineteen nucleotide variations were detected in the entire sequence, leading to the eight amino acid changes. The similarity of the buffalo cDNA sequence with the other species in GenBank was studied using BLASTN software which revealed that the Buffalo GDF-9 is 98% similar to Bos taurus, 97% to Ovis aries 85% to Homo sapiens, 84% to Sus scrofa, 83% to Mus musculus and 84% attus novergeticus.

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   1 M  A  L  P  N  K  F  F  L  W  F  C  C  F  A  W  L  C  F  P
   1 ATG GCG CTT CCC AAC AAA TTC TTC CTT TGG TTT TGC TGC TTT GCC TGG CTC TGT TTT CCT

  21 I  S  L  D  S  Q  P  S  R  G  E  A  H  T  L  A  R  T  A  L
  61 ATT AGC CTT GAT TCT CAG CCT TCT AGG GGA GAA GCT CAC ACT TTA GCT AGG ACT GCG TTG

  41 E  S  E  A  E  T  W  S  F  L  K  H  L  D  G  R  H  R  P  G
 121 GAA TCT GAG GCT GAG ACT TGG TCC TTC CTG AAG CAT CTA GAT GGG AGA CAC AGA CCT GGT

  61 L  L  S  P  L  L  K  V  L  Y  D  G  H  R  E  P  S  R  L  Q
 181 CTC CTT TCC CCT CTC TTA AAG GTT CTG TAT GAT GGG CAC AGG GAA CCC TCC AGG CTT CAG

  81 P  D  D  K  A  L  S  Y  M  K  R  L  Y  K  A  Y  A  T  K  E
 241 CCA GAT GAC AAA GCT TTG AGC TAC ATG AAG AGG CTC TAT AAA GCA TAC GCT ACC AAG GAG

 101 G  T  P  K  S  N  R  S  H  L  Y  N  T  V  R  L  F  T  P  C
 301 GGG ACC CCT AAA TCC AAC AGA AGC CAC CTC TAC AAC ACT GTT CGA CTC TTC ACC CCC TGT

 121 A  Q  H  K  Q  A  P  G  D  Q  A  A  G  T  L  P  S  V  D  L
 361 GCT CAG CAC AAG CAA GCT CCT GGG GAC CAG GCT GCA GGA ACC CTT CCA TCA GTG GAT CTG

 141 L  F  N  L  D  R  V  T  V  V  E  H  L  F  K  S  V  L  L  Y
 421 CTG TTT AAC CTG GAT CGT GTT ACT GTT GTG GAA CAT TTA TTC AAG TCA GTC TTG CTA TAT

 161 T  F  N  N  S  I  S  F  P  F  P  V  K  C  I  C  N  L  V  I
 481 ACT TTC AAC AAC TCC ATT TCT TTT CCC TTC CCT GTT AAA TGT ATA TGC AAC CTG GTG ATA

 181 K  E  P  A  F  F  S  K  T  L  P  R  A  P  Y  S  F  T  F  N
 541 AAA GAG CCA GCG TTT TTT AGC AAG ACT CTC CCT AGA GCT CCA TAC TCA TTT ACC TTT AAC

 201 S  Q  F  E  F  R  K  K  Y  K  W  I  E  I  D  V  T  A  P  L
 601 TCA CAG TTT GAA TTT AGA AAG AAA TAC AAA TGG ATT GAG ATT GAT GTG ACA GCT CCT CTT

 221 E  P  L  V  A  S  H  K  R  N  I  H  M  S  V  N  F  T  C  V
 661 GAG CCT CTG GTG GCC TCC CAC AAG AGG AAT ATT CAC ATG TCT GTA AAT TTT ACA TGT GTG

 241 K  D  Q  L  Q  H  P  S  A  R  D  S  L  F  N  M  T  L  L  V
 721 AAA GAC CAG CTG CAG CAT CCT TCA GCA CGG GAC AGC CTG TTT AAC ATG ACT CTT CTC GTA

 261 A  P  S  L  L  L  Y  L  N  D  T  S  A  Q  A  F  H  R  W  H
 781 GCG CCC TCA CTG CTT CTA TAT CTG AAC GAC ACA AGT GCT CAG GCT TTT CAC AGG TGG CAT
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Fig. 4 Nucleotide and amino acid sequence of GDF-9

3.8 Nucleotide and amino acid sequence of BMP-15 (Accession No. EF375880)

The nucleotide sequence and deduced amino acid sequence of BMP-15 is presented in the Fig. 8. Buffalo BMP-15 cDNA consist of an open reading frame of 1185bp coding for 394 aa residues of 44.94 Kd molecular weight. A multiple sequence alignment of buffalo BMP-15 was carried out with bovine BMP-15 sequence available in the GenBank (AB058416). Total eighteen nucleotide substitutions were detected at various positions out of which nine lead to the amino acid change.

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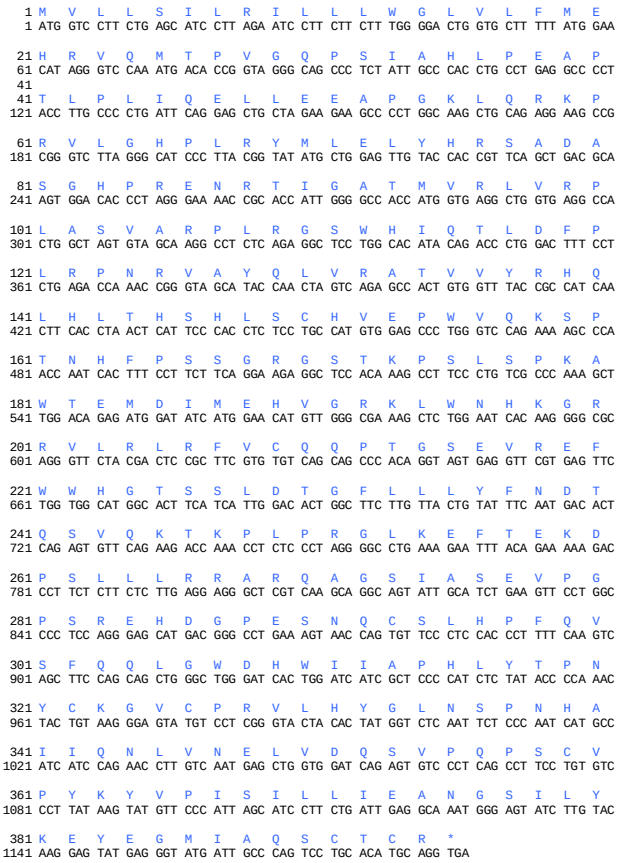

Fig. 5 Nucleotide and amino acid sequence of BMP-15

4. CONCLUSIONS

In conclusion, the results presented in the present study clearly demonstrate the expression of GDF-9, BMP-15 in Buffalo oocytes whereas Cx-43 was found to be expressed only in cumulus cells. Interestingly, HAS-2 was found to be expressed in both oocytes and cumulus cells. Data presented in the above study also demonstrate, effect of the culture time on the expression of genes involved in bidirectional communication during the IVM. However, the two Gonadotropins have similar effect on expression of developmentally important genes. Both GDF-9 and BMP-15 were found to be upregulated with the progression of IVM period. Thus, so far as the influence of gonadotropins (FSH and LH) on bidirectional mechanism is concerned we can interpret that for maintaining the HAS-2 expression both GDF-9 and BMP-15 acted as positive signals. Thus, at this point we may conclude that our result supports the synergistic role of GDF-9 and BMP-15 on HAS-2 expression.

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