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Effect of Growth Factors on Relative mRNA Abundance of COC Genes in Buffalo (Bubalus Bubalis)

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Abstract—*Study of effect of Growth Factors (EGF & IGF) on the genes responsible for bidirectional communication, may lead to important information in defining the oocyte competence. Present study was done to find out the temporal expression of GDF-9, BMP-15, HAS-2 and Cx-43 genes during in vitro maturation of Buffalo follicular oocytes and cumulus cells (COCs) under influence of Growth factors IGF(50ng/ml) and EGF(50ng/ml). Expression of these genes was analyzed using RT-PCR at 0, 8, 16 and 24 h of IVM. Analysis of expression of these genes revealed that both GDF-9 and BMP-15 were up regulated with the progression of IVM period till 24hrs of maturation. Interestingly, the oocytes in IGF/EGF supplement groups failed to undergo adequate cumulus expansion also. It may be speculated that oocytes under these experimental categories reflect an under maturation to undergo the normal pattern of transcriptional silencing at the end of IVM. The EGF/IGF groups resulted in up regulation of HAS2 up to 16 hrs and then down regulation till 24 hr of in vitro maturation. Our observation about the up regulation of HAS-2 up to 16 Hr IVM confirms many earlier reports indicating up regulation of HAS-2 with different media supplements during IVM. The reduction of expression beyond 16 Hr and after 24 Hr again hints at the fact that probably the maturation process in buffalo oocytes sets in early and continues at a faster rate. Cx-43 expression at 24 Hr of IVM either remained unaffected or moderately up regulated with EGF/ IGF. Finally, based on the observed results it could be interpreted that GDF-9 and BMP-15 maintained a positive relationship throughout the IVM period although at 24 hr the correlation value was significant (P<0.05).*

Keywords*: In vitro maturation, oocyte, GDF9 expression, bidirectional communication.BMP-15.*

1. INTRODUCTION

Cellular communication between oocytes, cumulus cells and the mural granulosa cells is mediated by various endocrine, autocrine, paracrine factors and gap junctional proteins. This communication is described as 'bidirectional' because it involves continuous exchange of intercellular signals between oocyte and its surrounding cells that ultimately impact fertilization potential of the oocytes [1], [2], [3]. 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 [4]. Expression of GDF-9, BMP-15,HAS2 and CX-43 is very crucial for developmental competence of oocytes. In this regard, study of effect of Growth factors EGF and IGF 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 EGF and IGF 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 in Buffaloes (Bubalus bubalis).

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 38.50^{o}C in 5%CO2 in air 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 50 ng/ml EGF (Sigma) 3. Control medium with 50 ng/ml IGF (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. [5] 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 (94^0C, 4 minutes), annealing (GDF-9 60^0C/30S; BMP-15 54^0 C/30S; HAS-2 54^0C/30S; Cx-43 55^0C/30S and 18S rRNA 62^0C/30S) and extension of 72^0C/30S for GDF-9, BMP-15, HAS-2, and Cx-43 and 72^0C/28S for 18S rRNA. Densitometry data for band intensities was generated using AlphaDigiDocTM AD-1201 software under Windows TM 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 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

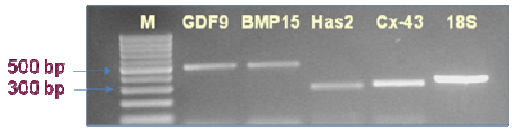

Fig. 1: Gel photograph showing expression of A.GDF-9 B. BMP-15 C. HAS-2 and D. Cx-43 and 18S rRNA

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

Expression profile of GDF9 and BMP 15 over different time intervals is presented in fig 2 which shows that the GDF-9 RA was least at the beginning (0Hr) of maturation which steadily increased up to 16 hrs and then drastically reduced at 24 Hr of maturation in IGF supplemented group whereas in EGF group, GDF9 expression continued to remain high till 24 hrs of IVM. With these observations we hypothesized that an assessment of GDF-9 RA at 16 Hr rather than 24 Hr may depict a more rational explanation about the effect of different media supplements on GDF-9 RA and its role in cumulus expansion during the course of IVM. The logical interpretation of the GDF-9 RA data in response to different media supplements as observed in the present study both at 16 and 24 Hr of IVM is supported by the earlier chronology of M-II progression data by Datta and Goswami, 1998 [6]. GDF-9 as a cumulus expansion stimulator (CEEF), one may speculate an increase in relative abundance of GDF-9 during IVM so that it could be correlated with the event of cumulus expansion. Earlier Datta and Goswami, 1998 reported that during IVM of buffalo oocytes with TCM-199 supplemented with pFSH and FBS resulted in attainment of M-II in more than 80% of the oocytes but in fact a sizeable percentage of these oocytes reached M-II at 20 hrs of maturation indicating that maturation process in buffalo

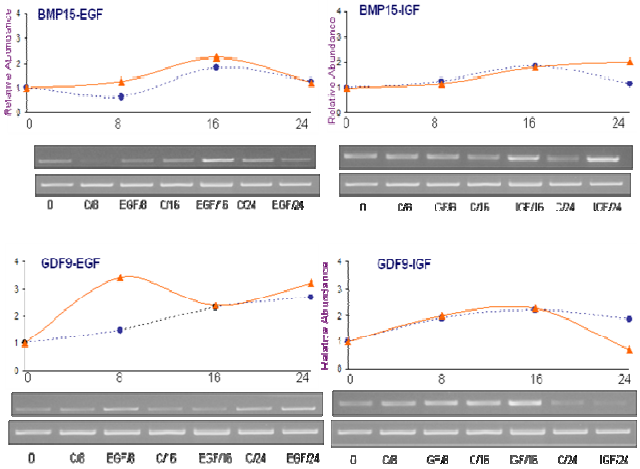

Fig. 2: Expression profile of GD9 and BMP15 in EGF and IGF media

oocytes is probably faster than other species. The conclusion that oocyte maturation in buffalo happens at faster rate, however, needs further investigation. In view of the discussion above we can conclude that the present observation of differential GDF-9 expression in buffalo oocytes, their time dependent expression pattern and the influence of different

media supplements on GDF-9 RA may provide vital clues to assess the developmental competence of oocytes during IVM and their subsequent development. BMP-15 RA was least at 0 Hr of maturation which steadily increased up to 16 Hr and then drastically reduced at 24 Hr of maturation in IGF supplemented group.

3.3 Expression of HAS-2 and Cx-43 at different hour intervals during IVM

In view of the apparent contradictory HAS-2 expression results obtained in the present study and more popular opinion about the up regulation pattern of HAS-2 during

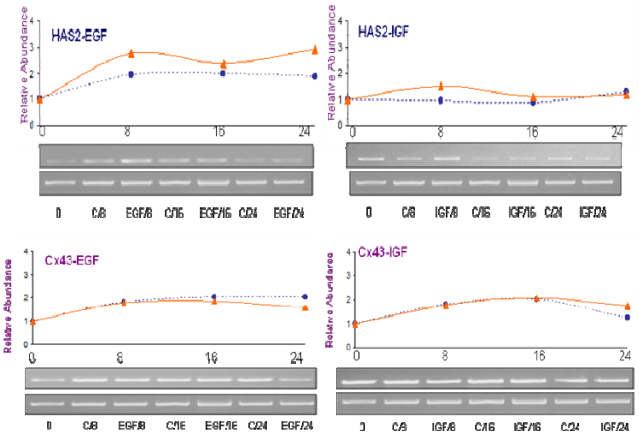

Fig. 3: Expression profile of HAS2 and Cx-43 in EGF and IGF media

IVM we asked the question that whether the HAS-2 expression in buffalo oocytes follow a definite time trend and whether it is a fact that at the end of 24 Hr when we measured the HAS-2 expression, its expression level was reduced physiologically? According to literature hyaluronan synthesis by cumulus cells was first detected 2-3 Hr after stimulation with FSH followed by further increase to a maximum between 4-10 Hr, and then declined and ceased by ~18 Hr [7]. Further, when transcription of HAS-2 mRNA was inhibited with actinomycin D at 6 Hr of IVM (when hyaluronan synthesis is maximum), net synthesis of HAS-2 was found to be decreased to a level equivalent to sustaining the maximum rate for only ~2 Hr [8] indicating that the half life of HAS-2 mRNA is short. To test this hypothesis with respect to buffalo oocytes we carried out the time interval study for the expression of HAS-2 in the course of IVM. The results are explained in This set of experiment was conducted with oocytes obtained from buffalo ovaries irrespective of their follicular diameter but restricted to only good quality oocytes selected on the basis of cumulus cell criteria.

Our result shows that the media supplements resulted in an increase of HAS-2 expression for initial 8 Hr and was followed by maintaining it to almost stable level up to 16 Hr beyond which it declined up to 24Hr; except with EGF where a continuous upregulation was observed. At all these time intervals the HAS-2 expression in respective media supplement groups was compared to respective control groups to differentiate between the physiological expression variation versus the effect of the particular media supplement. In human studies (ie et al., 2004) in this direction, it has been indicated that the expression of the HAS-2 in *in vitro* matured oocytes is positively correlated with their subsequent fertilization and development rate. Thus our present result with respect to cumulus expansion and HAS-2 expression again questions the reliability of a morphological criteria of cumulus expansion in determining the oocyte competence. Our observation about the upregulation of HAS-2 up to 16 Hr IVM explains the apparent contradiction of results as observed at 24 Hr and confirms many earlier reports indicating upregulation of HAS-2 with different media supplements during IVM. The reduction of expression beyond 16 Hr and after 24 Hr again hints at the fact that probably the maturation process in buffalo oocytes sets in early and continues at a faster rate.

However, the COCs treated with EGF showed higher expression level of HAS-2 even after 16 Hr and continued to be up regulated up to 24 Hr. EGF and EGF-like growth factors have been reported to stimulate HAS-2 and Cox-2 mRNA expression for maximal hyaluronan synthesis by COCs *in vitro* (Park et al., 2004). Role of EGF in stimulating cumulus expansion was reported by Downs et al., 1989. In this regard Salustri et al., 1999 have hypothesized that EGF generates an intracellular signal by interacting with tyrosine kinase receptors inducing HAS-2 transcription and ultimately producing more HAS-2 proteins in cumulus cells. In the same work a synergistic role of EGF and FSH has been indicated in the upregulation of HAS-2 although following different pathways. Various culture methods of cumulus cells and cumulus cell-oocyte complexes have provided evidence that hyaluronan synthesis is under the control of several other factors apart from FSH and EGF.

Connexin 43 (Cx-43) is a gap junctional protein found between cumulus cells and is required for the exchange of small molecules from cumulus cells to oocytes (Grazul-Bilska et al., 1997). This protein is also cited as an important mediator involved in the cross talk between oocytes and surrounding cumulus cells [9]. In the present study we wanted to experiment on the expression pattern variation of Cx-43 along with HAS-2 as mediators in bidirectional communication in the cumulus to oocytes route in response to different media supplements used With respect to the Cx-43 expression in oocytes during IVM the overall contradiction of results prompted us to suspect the maturation dynamics which might be typically different for buffalo. As stressed earlier under the sections of GDF-9/BMP-15/HAS-2 and in view of the M-II percent data as observed by Datta and Goswami, 1998 we were keen to workout the expression of Cx-43 with different supplements at 0, 8, 16, and 24 Hr of IVM and the results are presented in fig.3. It could be seen that as a general trend in all experimental groups as well as in control groups

the expression pattern revealed an upward trend from 0-6 hr and further up to 16 Hr followed by a downtrend up to 24 Hr. Our result was in agreement with the similar study in bovines where Cx-43 mRNA expression was found to be increased between 0-6 Hr and then decreased up to 24 Hr during the time course of IVM [10]. Regarding EGF and IGF the expression at either time intervals were almost stable. In conclusion, we can say that although expression of Cx-43 is indispensable for the growth and differentiation of the oocytes our results do not support the hypothesis that it could be used as a developmental marker for the oocytes competence. Rather, based on our result we would interpret that Cx-43 is physiologically important for maintaining gap junctional communication between the oocytes and surrounding cumulus cells. Further its expression behavior over 24 Hr of IVM in buffalo oocytes again hints at the possible faster rate of maturation in this species which however needs more detailed investigation.

4. ACKNOWLEDGEMENTS

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