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Expression of *PTX3, GHR* and *GDF9* genes in cumulus cells and oocytes in relation to developmental competence of *Bos indicus*

H DEV[1], D J DUTTA[1]✉, P BORAH[1], H RAJ[1], I HUSSAIN[1] and J SAIKIA[1]

College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam 781 022 India

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Developmental competence of oocytes has always been a major concern in Assisted Reproductive Technology (ART) efficiency. Ability of the oocyte is reflected in it fulfilling the process of undergoing maturation, successful fertilization, reaching the stage of blastocyst and finally in yielding a viable and healthy progeny (Assidi *et al*. 2008). The terminal steps of folliculogenesis and meiotic maturation primarily depends on the establishment of a fruitful molecular crosstalk between the oocyte and surrounding follicular cells. Prediction of the developmental potential of harvested cumulus-oocyte complexes (COCs) is crucial for an effective oocyte selection (Gilchrist *et al.* 2008).

Expression pattern of certain genes in cumulus cell (CC) is suggested as a non-invasive analytic method of ART to predict competence of oocytes. Several genes were reported to be up- or down-regulated in the cumulus cells of competent oocytes (Li *et al.* 2000, Salustri *et al.* 2004, Caixeta *et al.* 2009) with species differences. A number of genes have been identified that are differentially expressed in oocytes and cumulus cells, expression pattern of which could be associated with competence of oocytes (Caixeta *et al.* 2009). Among those, Pentraxin 3 (*PTX3*) plays a key role in the organization of cumulus oophorus extracellular matrix and in fertilization *in vivo* (Salustri *et al.* 2004). Growth differentiation factor 9 (GDF9) plays a critical role in granulosa and theca cell growth as well as in differentiation and maturation of the oocytes. It is required for ovarian folliculogenesis; it promotes primordial follicle development, and also stimulates granulosa cell proliferation. Growth Hormone Receptor (GHR) helps in differentiation and replication (Izadyar *et al.* 1998) in addition to the modulation of steroidogenic gene expression (Doldi *et al.* 1996). Consequently, all these genes can be considered as the candidate genes for studying their effects on cumulus cells of oocytes and the resultant information may be utilized for successful prediction of developmental competence of bovine oocytes.

The present study was carried out in the Department of Veterinary Physiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati with prior approval from the Institutional Animal Ethics Committee. Slaughterhouse cattle ovaries were collected and transported to the laboratory in normal saline within 2 h. Cumulus- oocyte- complexes (COCs) were collected by aspirating surface follicles of 2-8 mm diameter with standard procedure and graded morphologically. Only good quality COCs with three or more compact layers were selected for the present study. *In vitro* maturation (IVM) of COCs had been carried out in Modified TCM-199 with 200 mM L-glutamine solution, 10% FBS, 0.8 M sodium pyruvate, and 50 µg/ml gentamicin and 50 µM cysteamine supplemented with porcine-follicular stimulating hormone (5 µg/ml), 10% v/v follicular fluid, 1 µg/ml 17-β estradiol at 38.5°C in a humidified atmosphere of 5% CO_2 for 24 h. For *in vitro* fertilization, frozen bovine semen (2 straws each) was prepared for capacitation with swim-up technique using B.O. washing medium to make a final concentration of 2×10^6 sperm/ml. *In vitro* matured oocytes were co-incubated with spermatozoa in B.O. fertilization medium at 38.5°C, 5% CO_2 in air and saturated humidity for 20-22 hr. The expected zygotes were washed in culture (IVC) medium (viz. mCR2aa containing 5% FBS and supplemented with 2% essential amino acids (v/v), 1% non-essential amino acids (v/v), 1% α- glutamic acid, 0.3% BSA and 0.05 µg/ml gentamicin sulphate). Then zygotes were placed into IVC droplets and covered with mineral oil and incubated. After 48 hrs culture, the cleavage up to 2-8 cells was recorded. Subsequent embryo development was observed under a microscope every 24 hrs following insemination. The medium was replaced with fresh one in every 48 h of culture. The study was carried out on different dates with replicates of 10 times.

After *in vitro* maturation of oocytes in the maturation medium, few oocytes would undergo *in vitro* fertilization, and the rest oocytes and cumulus cells to be used for gene expression analysis were separated by repeated pipetting.

Present address: [1]Assam Agricultural University, Khanapara, Guwahati, Assam. ✉Corresponding author e-mail: duttadj@hotmail.com

Table 1. Gene transcripts, primer sequences and resulting fragment size

Target	Sequence of nucleotide	Fragment size (bp)
GDF 9	Forward: AGTGGGACAACTGGATTGTG	103
	Reverse: GTGTGAACTGGAGAGCCATAC	
GHR	Forward: CGTCTTATACCTCTGTGTGGAC	110
	Reverse: GGGTGGATCTGGTTGTACTATG	
PTX3	Forward: GCACCTGGGATTCAAAGAAAG	88
	Reverse: ACATGACCTGTGGCCATATC	
GAPDH	Forward: GTCATCATCTCTGCACCTTCTG	99
	Reverse: GGAGGCATTGCTGACAATCT	

A total of 20-25 number of oocytes were subjected to mRNA isolation, subsequent complementary DNA (cDNA) synthesis and gene expression analysis.

Cumulus cells removed from oocytes were transferred into 1.5 ml tubes (Eppendorf SRL, Milan, Italy) with a minimal amount of Trizol. Cumulus cells were isolated by repeated pipetting and washed with Phosphate Buffered Saline (PBS). Cumulus cells from different COCs were pooled together in order to have enough material to enable the direct comparisons of different procedures. The number of cells for each pool was determined by measuring the amount of DNA extracted from an aliquot of each pool with Wizard® Genomic DNA Purification Kit (Promega Italia, Milan, Italy) and quantified using the NanoDrop ND-1000 spectrophotometer (EuroClone S.P.A., Milan, Italy). A total of 20-25 matured oocytes as well as their respective cumulus cells were picked up to purify the RNA. Total RNA was isolated using Trizol method and stored at -80ºC till use for cDNA preparation. Reverse transcription of the RNA extracted from oocytes was performed using RevertAid™ M-MuLV Reverse Transcriptase. The primers used for Real-Time PCR were designed using the nucleotide sequence of the genes in NCBI Gene bank database by Primer 3 software. Primers were commercially synthesized by Integrated DNA Technologies (IDT). The primer sequences used in the study are listed in Table 1.

The quantification of all gene transcripts was carried out by real time quantitative RT-PCR. Three replicate PCR experiments were conducted for all genes of interest using oocytes collected from the four experimental pools. Real-Time PCR was performed using Step-One Plus real time amplification instrument (Applied Biosystem by Life Technology, USA). Reactions were performed using Maxima SYBR green/ROX qPCR Master Mix as a double-stranded DNA-specific fluorescent dye, with the appropriate primer set. All samples were measured in triplicates. Reactions were performed in 20 µl reaction mixture and a Non-Template Control (NTC).

The following real time PCR protocol was employed for all investigated factors: initial denaturation at 95°C for 10 min, denaturation at 95°C for 15 sec, annealing/extension at 60°C for 1 sec for all genes for 40 cycles and the last cycle at 95°C for 15 sec, at 60°C for 1 sec and 95°C for 15 sec for 1 cycle and continuous fluorescence measurement, and a final cooling down to 40°C. Initially, specificities of all assays were checked by conventional PCR using thermal cycler and size of the amplicon was checked in agarose gel electrophoresis. After obtaining a single band, the optimization of Real-Time PCR was performed. The optimal primer concentration for the primer pair was determined by performing duplicate Real-Time PCR assays at different primer concentrations over the range of 5 to 20 pmol/µl. From the fluorescence data and melting curve analysis, the optimal primer concentration was obtained. The melt curve assisted in establishing primer specificity and the threshold cycle value (Ct) helped in determining sensitivity of the assay. If the melt curve indicated non-specific product, the annealing temperature was increased. The lowest Ct value defined the optimal primer concentration.

The relative expression of genes was determined by carrying out Real-Time PCR of target and normalization genes. Standard curves were generated for target and internal control genes. The relative quantification of target genes expression was calculated using $2^{-\Delta\Delta Ct}$. The threshold cycle (Ct) values were based on triplicate measurements and each experiment was repeated twice. The quantification values obtained for target genes in control were used for calibration and were arbitrarily set to 1 and 0 for linear and log graph types, respectively. The data analysis was carried out by StepOne® software v 2.2.2. Data obtained in the present experiment were analyzed statistically using SAS Enterprise Guide 4.3. GAPDH was selected as the housekeeping gene for performing the real time assay for which target genes were optimized.

In the present study, the relative quantified values obtained for target genes (*GDF9, GHR,* and *PTX3*) in the control mature cumulus (MC) cells was used for calibration and was considered as unit (one). The relative quantification (RQ) values of *GDF9* (Table 2) indicates that the expression of *GDF9* gene was up-regulated in Mature Oocyte (MO) and Immature Oocyte (IO) compared to the reference control, i.e. MC. The expression pattern of *GDF9* was relatively more in mature oocytes compared to immature oocytes. Oocyte-secreted factors like *GDF9* enhance oocyte developmental competence during *in vitro* maturation, either in their native form as an uncharacterized mix of growth factors secreted by the oocyte, or as recombinant exogenous *BMP15* and *GDF9*. Growth and differentiation factor 9 (*GDF9*), a member of the transforming growth factor-β (TGF-β) super family, was the first known oocyte-specific gene responsible for cumulus expansion. The *GDF9* gene regulates several key granulosa cell enzymes involved in cumulus cells expansion and promotes the acquisition of oocyte developmental competence acting as a paracrine factor. The expression of *GDF9* downstream target genes in the cumulus cells reflects *GDF9* activity and predicts oocyte health as well as the grade of the resulting embryos (Ferrari *et al.* 2010). Transcripts that are over-expressed in

oocytes are involved in the processes leading to meiotic maturation. Regassa *et al.* (2011) found considerably higher (more than 1024-fold change) and exclusive expression of *GDF9* in oocyte compared to CCs. Caixeta *et al.* (2009) observed that oocyte–specific gene *GDF9* expression was not affected by follicle size and similar transcript level of expression was found in bovine oocytes with different developmental competence. Expression of genes coding for *GDF9* and *BMP15* prevents cumulus cell apoptosis and may influence the process of oocyte maturation. In the present study, expression profile of *GDF9* was most markedly up-regulated in the mature oocytes compared to cumulus cells. Whether this discrepancy in expression is a reflection of the type of vitrification or a species-specific difference, that needs further investigation.

The RQ values of *GHR* (Table 2) indicates that the expression of *GHR* gene was up-regulated in oocytes irrespective of the developmental stages, though *in vitro* matured oocytes showed higher transcript level than immature oocytes. Receptors for growth hormones are required to mediate the action of the legend, which has a role in the IVM of bovine oocytes (Bevers and Izadyar 2002). Prada *et al*. (2010) reported that there was evidence for a role for GH in oocyte maturation in a variety of mammalian species. Growth hormone has stimulatory effects on oocyte maturation in cattle, rats, horses, pigs and rabbits (Yoshimura *et al.* 1993, Apa *et al.* 1994, Izadyar *et al.* 1997, Marchal *et al.* 2003). The expression of *GHR* in the fully-grown bovine oocytes also points toward a possible role of GH during embryo development following fertilization of the matured oocytes. However, Caixeta *et al.* (2009) found no difference in the expression of *GHR* in bovine oocytes from follicles of different sizes.

The RQ values of *PTX3* (Table 2) indicates that, in respect to cumulus cells, the transcript level of *PTX3* is up-regulated in Mature Cumulus (MC) compared to Immature Cumulus (IC). In oocytes, no marked changes in the transcript level of *PTX3* were observed. These findings were in conformity with the statement of Zhang *et al.* (2005) that changes in the expression levels of 160 genes including *PTX3* in human cumulus cells might be indicative of the quality of their enclosed oocytes. Expression of *PTX3* was lower in mature than immature cumulus. The *PTX3* gene acts to stabilize the expanded cumulus and is regulated by *GDF9*. Fertilization *in vivo* is very low in *PTX3* knockout mice, whereas it was normal *in vitro* (Varani *et al.* 2002).

Table 2. Relative quantification (RQ) values of *GAPDH*, *GDF9*, *GHR* and *PTX3* gene expression in different samples by qPCR

Sample	Target	RQ
Immature cumulus (IC)	*GDF9*	#######
	GHR	#######
	PTX3	#######
Immature oocyte (IO)	*GDF9*	#######
	GHR	#######
	PTX3	0
Mature cumulus (MC)	*GDF9*	1
	GHR	1
	PTX3	1
Mature oocyte (MO)	*GDF9*	#######
	GHR	#######
	PTX3	#######

Note: Mature cumulus (MC) cells were taken as control.

On the basis of the present study, it was concluded that *GDF9* and *GHR* genes of bovine oocytes may be considered as candidate marker genes for embryonic development in Assisted Reproductive Technologies (ARTs). Sustainable growth and expansion of bovine cumulus cells depended on inherited *PTX3* gene and hence it could be considered as a marker gene for cumulus cell development.

SUMMARY

The present study was conducted to evaluate the level of expression (mRNA) of specific developmental marker genes, viz. Growth Differentiation Factor 9 (*GDF9*), Growth Hormone Receptor (*GHR*) and Pentraxin 3 (*PTX3*) in bovine cumulus cells and oocytes in relation to *in vitro* developmental competence. Good quality Cumulus-Oocytes- Complexes (COCs) were selected for *in vitro* maturation and fertilization. The mean percentage of *in vitro* fertilization performance was 59.84 ± 4.28. The relative quantification values of *GDF9* were 2.38, 98.13, 1 and 132.58; *GHR* were 4.31, 1.47, 1 and 43.58; *PTX3* were 0.28, 0, 1 and 0.5 in immature cumulus cells, immature oocyte, mature cumulus cells and mature oocyte respectively when compared to the reference control. The expression of *GDF9* gene was found to be up-regulated in oocytes compared to cumulus cells. The expression pattern of *GDF9* was relatively more in *in vitro* matured oocytes compared to cumulus groups. The expression of *GHR* gene was up-regulated in oocytes. The relative abundance of *PTX3* was slightly up-regulated in mature cumulus cells as compared to immature counterparts. Thus, *GDF9* and *GHR* genes could be considered as embryonic developmental markers for bovine oocytes, while *PTX3* gene for bovine cumulus cells.

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