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In vitro Maturation of Buffalo Oocytes and Expression of Selected Biomarker Candidate Genes

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ABSTRACT

Background: Number of genes expressed during *in vitro* maturation (IVM) of which selected genes can be used as the potential biomarkers of oocyte competence. Hence, this study was planned to evaluate selected gene expression of *GDF9, HAS2*, *SPRY, ARHGAP22, COL18A1 and GPC4* in IVM and immature cumulus oocyte complexes (COCs).

Methods: The COCs were recovered from slaughter origin ovaries of buffaloes. Of which first three grade COCs were randomly allotted in immature (IMT; n=217) and IVM group (n=272). IVM of COCs was performed under drops of BO-maturation media in CO_2 incubator at 39.0°C for 24 hours. The expression of genes was evaluated using qPCR and the relative expression of each gene was calculated using the ΔΔCt method with efficiency correction. The logarithmic transformation of fold change of each candidate genes in the IVM group was computed against the IMT group using the Ct values.

Result: The expression obtained for IVM group of earlier reported up-regulated genes (GDF9, HAS2, SPRY1) was higher (upto 10xfold) compared to the IMT group. Relatively lower expression was observed for the rest of the three candidate genes (ARHGAP22, COL18A1, GPC4) in the bovine transcripts of oocyte which were earlier also reported as down regulated.

Key words: Buffaloes, Gene expression, *In vitro* maturation, Oocyte.

INTRODUCTION

Morphological assessment is the common practise to assess successful *in vitro* maturation (IVM) based on the number of layers and compactness of cumulus, homogeneity of the ooplasm and extrusion of the first polar body (Das *et al.* 1996). However, these morphological evaluations are subjective and with limited reliability enough to act as the sole criteria for the evaluation of oocyte competence. Understanding the gene expression patterns in Cumulus Oocyte Complexes (COCs) is critical to provide insights into the complex regulatory networks and for the identification of candidate genes relevant to the undergoing biological processes. Six genes were selected in the present study from the different biochemical pathways *viz*., *sprouty RTK signalling antagonist 1*(SPRY1; Melo *et al*. 2017), *Rho GTPase activating protein 22* (ARHGAP22; Melo *et al*. 2017); *collagen type XVIII alpha 1 chain* (COL18A1; Melo *et al*. 2017) *glypican 4* (GPC4; Melo *et al*. 2017) *growth differentiation factor 9* (GDF9; Kathirvel *et al*. 2013) and *hyluronansynthetase 2* (HAS2; Bhardwaj *et al*. 2016) based on their physiological roles as candidate biomarkers for oocyte competence from previous studied relating to gene expression patterns of bovine oocyte development. The GDF9 plays an essential role during follicle maturation through actions on granulosa cells (Kathirvel *et al*. 2013); HAS2, mRNA expression is considered as an important prerequisite for extrusion of oocyte from follicle at the time of ovulation and sperm-oocyte interaction mediated by hyaluronan (Bhardwaj *et al*. 2016); downregulation of SPRY1 is associated with angiogenesis in endothelial cells and increased cell proliferation (Melo *et al*., 2017), ARHGAP22 is responsible for *Rho GTPase* activating protein 22 and its downregulation is associated with decrease in requirement of angiogenesis as follicle grows in preparation of ovulation (Melo *et al*., 2017); the lower expression of COL18A1 gene that encodes *type XVIIIα1 collagen* is important for the impaired follicle wall resistance necessary for its rupture during ovulation (Melo *et al*., 2017); GPC4 gene is member of the *glypican 4* family and plays key role in regulation of fibroblast growth factors (FGF), bone morphogenic protein and growth differentiation factors signalling and also mediates the expression of cumulus-specific matrix genes, HAS2 and TNFAIP6 (Melo *et al*., 2017).

The present study envisages to evaluate and compare the relative expression of selected genes in COCs obtained

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from size-incompetent COCs (Immature) and size-competent COCs (IVM group) by qPCR in the domestic water buffalo.

MATERIALS AND METHODS

The experiment was carried out between January and March 2020.

Ovarian collection and grading of oocytes

Ovaries of apparently healthy adult buffaloes with unknown reproductive history slaughtered at slaughterhouse belonging to, Ahmedabad Municipal Corporation, Ahmedabad, Gujarat, India were collected in thermos having 37°C warm normal saline (NS:0.9% NaCl) supplemented with antibiotics (1,00,000 IU penicillin and 100 mg streptomycin/L). The ovaries were transported in a thermos from the slaughterhouse to the laboratory within 45 min. Ovaries were processed to remove extra tissues and washed three-four times with warm sterile NS in the ovarian processing arena of the IVF laboratory and kept in a sterile glass beaker containing warm sterile NS. A stereoscopic microscope (Nikon SMZ-2B, Tokyo, Japan) was used to identify COCs which were transferred into a 35×10-mm (Tarson®, Tarsons Products Pvt Ltd, India) disposable plastic petri-dish containing pre-warmed drops of 100µl of BO wash (IVF bioscience, Falmouth TR11 4TA, United Kingdom). The COCs were washed at least three times in BO wash drops and graded into four categories according to the number of layers viz, Grade I oocyte has more than 5 layers of cumulus with evenly granulated ooplasm, Grade II 3-4 layers of cumulus, Grade III 1-2 layers of cumulus with *corona radiata* and Grade IV, denuded oocyte with shrunken or distorted cumulus layer. In the present study, only the first three grades of oocytes were included (Das *et al*. 1996).

Experiment design

The first three graded COCs were selected either for immature (IMT) or IVM treatment. *In vitro* maturation of COCs were performed keeping in pre-warmed 90 µl drops of BO IVM media in 35 mm petri-dish. The BO media drops with COCs covered with silicon oil in 35 mm petri-dish were incubated for 24 h in a CO_2 incubator (5% CO_2) at 39.0°C. The IVM procedure was performed as per the Manufacturer's protocol (IVF bioscience, Falmouth TR11 4TA, United Kingdom). No such treatment was given to IMT COCs.

The IMT and IVM group COCs each were further transferred separately to a 50µl droplet of phosphate-buffered saline (PBS) and then to 200µl of 0.25% trypsin for two minutes for digestion of cumulus mass. Thereafter washed in 100µl of PBS to inhibit the action of trypsin. After cumulus digestion, oocytes were transferred to a 2ml collection tube containing 500µl RNAlater (invitrogen™, Thermo Fisher Scientific Pvt. Ltd, Waltham, Massachusetts, U.S.) and stored at -80°C until RNA extraction.

Assessment of nuclear maturation

Nuclear maturation was confirmed on 10% representative COCs of IMT and IVM groups using the Hoechst 333224 (Sigma-Aldrich, SAFC, St. Louis, Missouri, United States) nuclear staining method. The cumulus mass of COCs of IMT and IVM groups were removed by gentle pipetting and were stained with Hoechst 333224 using a dye solution (10µl/L). The oocytes were incubated for 10 min at 37°C and evaluated under a 40X epifluorescence microscope (Olympus, BX53 Upright Microscope, Shinjuku, Tokyo, Japan), with a filter wavelength of BP330-385 nm. The oocytes which were with germinal vesicle (GV) stage or germinal vesicle breakdown (GVBD) are considered as an immature group of oocytes. After Maturation, the oocytes shows different stages of cell cycle *i.e.* anaphase, telophase or polar body (Metaphase II) in perivitelline space it confirmed nuclear maturation for IVM.

Total RNA extraction

Total RNA was extracted from 5 sets of pooled oocytes (n~40) of the IMT Group as well as the IVM group using RNeasy Mini Kit (QiagenPvt. Ltd Venlo, Netherlands) according to the manufacturer's instructions. Thereafter RNA samples were evaluated using a QI Axpert Instrument (Qiagen Pvt. Ltd Venlo, Netherlands) and Qubit 4.0 (Thermo Fisher Scientific Pvt. Ltd, Waltham, Massachusetts, U.S. A) using the Qubit™ RNA HS Assay Kit (Invitrogen™ Thermo Fisher Scientific Pvt. Ltd, Waltham, Massachusetts, U.S.A.). Total RNA was also analysed using Agilent 2100 Bioanalyzer and appropriate RIN values were obtained.

cDNA synthesis

Samples with 260/280 ratio integrity ~2.0 were subjected to reverse transcription reaction with high capacity cDNA synthesis kit (Applied Biosystems, Thermo Fisher Scientific Pvt. Ltd, Waltham, Massachusetts, U.S.) as per the manufacturer's instructions. PCR amplification of the target genes as discussed before was done in an Applied Biosystem thermal cycler PCR machine with an amplification reaction mixture of a total 10µl volume consisting of 5µl of emerald master mix (Applied Biosystems, Thermo Fisher Scientific Pvt. Ltd, Waltham, Massachusetts, U.S.), 0.5µl each forward and reverse primer of 0.5µM concentration, 2µl of NFW and 2µl c DNA. No template control (NTC) was maintained for each set of primers and PCR products were analysed by electrophoresis 2% agarose TAE gel with 100bp ladder (Applied Biosystems, Thermo Fisher Scientific Pvt. Ltd, Waltham, Massachusetts, U.S.).

qPCR based expression analysis of candidate genes for confirming maturation of oocytes

Six genes as listed earlier were selected from the literature (Melo *et al*. 2016; Bhardwaj *et al*. 2016; Kathirvel *et al*. 2013) according to their biochemical pathway (cell cycle) and possible role in oocyte competence. Real-time RT-PCR analysis was performed using Fast SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific Pvt. Ltd, Waltham, Massachusetts, U.S.A.). Reactions were performed in a volume of 10µl. The PCR cycling conditions

were as follows: 95°C for 20s; 50 cycles of denaturing at 95°C for 3s and annealing at 58°C for 30s. Primer sequences, fragment size, annealing temperature and primer concentrations for each gene are listed in Table 1. Reactions were performed in triplicates for each gene. The expression of the endogenous control genes 18s ribosomal RNA (18s) and Tubulin (tuba) were assessed (Sui *et al*. 2020), but only 18s expression was used to normalize target genes due to its higher expression stability than a tuba. The relative expression of each gene was calculated using the ΔΔCt method (Livak and Schmittgen, 2001) with efficiency correction**.** The logarithmic transformation of fold change (log2FC) of each candidate gene in the IVM group was computed against the IMT group based on the observed Ct values. Appropriate standard deviation was also depicted based on the observed deviations among the triplicates.

Statistical analysis

The number of ovaries collected and the total COCs distributed among two groups *i.e.* IVM and immature oocyte groups in the present study were analysed using the "student's t-test". All statistical analyses were performed using SPSS 26 (IBM SPSS software Bengaluru, India).

RESULTS AND DISCUSSION

Five hundred and ninety-two COCs were aspirated from 234 buffalo ovaries from the slaughterhouse. Out of which 217 and 272 COCs of the first three grades (n = 489) were randomly allocated to IMT and IVM groups, respectively. Between the groups, within grades, or in total COCs no difference was observed (p< 0.01).

Using the list of genes (Table 1) with an expression change of >=2.5 fold, we selected three up-regulated genes (GDF9, HAS2 and SPRY1) and three down-regulated genes (ARHGAP22, COL18A1 and GPC4) as a candidate for biomarkers of COCs quality. These six genes were selected subjectively considering the physiological function of each gene as the main criterion. The expression change for each of the selected gene was validated by qPCR as it is the most adequate technique to measure the expression of candidate genes during IVEP routine practice. For the experiment 18s and tuba were used as housekeeping gene controls due to their documented performance as stable control for bovine COCs. Further, 18S was found to be more stable than tuba during qPCR gene expression analysis based on its consistent expression levels across the samples and run.

The expression profile of six genes obtained with RT-qPCR is presented in Fig 1. The RT-qPCR analysis of IMT and IVM groups confirmed the expression of reference (18S) as well as of selected gene transcripts. Fold change values of differential gene expression in oocytes were detected by qPCR, for GDF9, SPRY1, HAS2, ARHGAP22, COL18A1 and GPC4 (Fig 1). In our study two genes *i.e.* COL18A1 and ARHGAP22 has shown (with a fold change of ≥2.5) fold up regulation in IVM group compare to IMT

Table 1: List of primers used to analyse Maturation of oocytes.

Gene name	Primer Sequence	Expression in Mature oocytes	Amplicon Size	Annealing Temperature (ºC)	primer concentration (µmol)
GDF-9: Growth differentiation factor 9	F: 5'-TGCTCAGGCTTTTCACAGGTGGCA-3' R: 5'-GACGGGACAATCTTAC ACCCTCAG-3'	Up-regulated	131	67	0.01
HAS-2: Hyaluronansynthetases	F: 5' -ATAAATGTGGCAGGCGGAAGAAGG-3' 'R: 5' -GTCTTTGTTCAAGTCCCAGCAGCA-3'	Up-regulated	144	64	0.01
SPRY1: Sprouty RTK signalling antagonist 1	F: 5' -CATGTGCTTGGTCCAAGGGCATCTT-3' R: 5'-TGTGACTGTGAACAGGAGCAAGGA-3'	Up-regulated	93	65.7	0.01
GPC4:Glypcian 4	F: 5'-TGGTGAATCCCCACAACCCAGTGTA-3' R: 5'-TCTCAGCCACCATCAGCATAGCAT-3'	Down-regulated	192	63.83	0.01
COLI8A1: Collagen type XVIIalpha 1chain	F: 5'-ACAGCAACGATTTGTGGACTCTG-3' R: 5'-ACGTCTCCTTTGTCACCCTTGAGT-3'	Down-regulated	94	63.78	0.01
ARHGAP22: Rho GTPase activatingprotein 22	F: 5'-AGCAGAGAACATCATGAAGGGCT-3' 'R: 5'-ACCTGGGTCCCTTGCAGAGAAATA-3'	Down-regulated	127	63.42	0.01
18S : 18S ribosomal RNA	F: 5' -GTAACCCGTTGAACCCCATT-3' B: 5' -CCATCCAATCGGTAGTAGCG-3'	Housekeeping genes [Endogenous control]	112	63.78	0.01
Tuba :Tubulin	F: 5' -ACTACCAGCCACCCTCTGTGTC-3' 'B: 5' -GCACAAACGCACGATTACA-3'		82	63.65	0.01

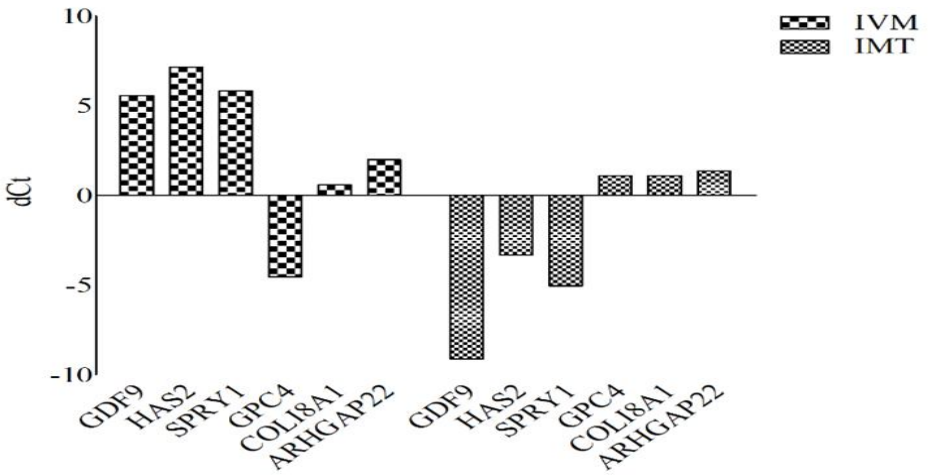

Fig 1: RT-PCR Expression analysis of the candidate genes in the IVM group compared to the IMT group (dCt – deltaCt normalized by the Ct value of endogenous control 18s in each group; IVM: *In Vitro* Maturation treatment group; IMT: Immature treatment group).

group which were down-regulated genes than in earlier reference studies (Melo *et al*. 2017). Further GPC4 was down regulated (with a fold change of ≥2.5) in IVM group which is in accordance to Melo *et al*. (2017). With reference to our study, the three up regulated genes GDF9, HAS2, SPRY1 demonstrated up regulation (≥8x fold) in comparison to IMT which is accordance to previous studies (Kathirvel *et al*., 2013; Bhardwaj *et al*., 2016; Melo *et al*., 2017).

The genes selected from the literature in our study showed a similar pattern of expression in other breeds of bovine (*B. indicus* × *B. taurus* crossbred) which indicates that these genes are in high correlation with oocyte developmental competence. After qPCR analysis, on the basis physiological role out of six genes selected in this study, three are up-regulated (GDF9, HAS2, SPRY1) and three are down-regulated (COL18A1, GPC4, ARHGAP22). Each gene has a distinct and specific role. The up-regulatory gene GDF9 plays an essential role during folliculogenesis (Gilchrist and Thompson, 2007) and is in relative abundance in cumulus cells (CC) which remains stable during IVM up to 12h of maturation and decreases significantly between 12h to 24h of maturation, hence plays a key role in the selection of dominant follicles (Kathirvel *et al*., 2013).

The up-regulated expression of SPRY1 resembles the expression pattern of the FGF family. FGF11 is also a negative regulator of steroid synthesis and granulosa cell survival and is expressed in mouse oocytes and embryos (Zhong *et al*. 2006). The SPRY family members are strictly expressed in mammals during initial embryo development (Melo *et al*. 2017).

The down-regulation of ARHGAP22 is associated with a decrease in the requirement of angiogenesis as the follicle grows in preparation for ovulation. In oocyte transcript, the low regulation of COL18A1 gene encodes *type XVIIIα1 Collagen*, which is important for the impaired follicle wall resistance necessary for its rupture during ovulation (Melo *et al.* 2017), as it is present in the basal lamina, which is responsible for degradation of collagen fibres during rupture of the follicle.

In our study, GPC4 was less expressed in oocyte transcript and HAS2 expression was higher (Fig 1). This gene is formed by heparin sulphate proteoglycans that are covalently attached to the cell membrane via a glycosyl phosphatidylionsitol anchor (De Cat and David, 2001). Therefore, it was considered GPC4 to be a good embryo quality marker candidate because its regulation was observed in oocyte developmental competence and it mediates the cumulus-specific matrix genes HAS2 and TNFAIP6 (Melo *et al*., 2017).

CONCLUSION

In this study the expression profile of differentially expressed genes were explored in between IMT and IVM oocyte groups harvested from buffalo's ovaries. During maturation of COCs *in vitro*, significantly expressed genes were considered as biomarkers of oocyte competence which is needed to ascertain IVEP embryo quality. The expression of three down-regulated (*COL18A1, GPC4, ARHGAP22*) and three up regulated (*GDF9, HAS2, SPRY1*) candidates were validated by qPCR as a probable marker of oocyte quality and competence for ART. In the present study, there was relatively lower expression of three genes in bovine transcripts of oocytes which were also down regulated in reference studies on cumulus cells.

Compliance with ethical standards

Statement of animal rights

All applicable international, national and institutional guidelines for the care and use of animals were followed.

Conflict of interest statement

The authors pronounce that they have no irreconcilable situation.

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