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COMPARATIVE ANALYSIS OF GENES RELATED TO QUALITY OF OOCYTE IN BUFFALOES (*Bubalus bubalis*) AND CAMELS (*Camelus dromedarius*) UNDER IN VITRO CONDITIONS

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SUMMARY

The quality of oocyte is one of the main determinants of in vitro embryo production success. Different approaches have been applied for analyzing oocyte qualities particularly gene expression analysis, which represents a new tool for selecting competent ones. Thus, the present study aimed at studying gene expression of morphologically good and poor immature oocytes of buffaloes and camels. A total of 360 COCs of buffaloes and camels (n= 180 each) were selected to run this study. Out of which 90 were pooled in three replicates with 30 oocytes in each of the two groups (immature good and poor grade oocytes) were used for gene expression analysis after cumulus cells denudation. Total RNA was isolated from all samples using PicoPureTM RNA isolation kit, and then converted into cDNA via reverse transcription kit. Quantitative Real-Time PCR was performed using specific primers for a set of selected candidate genes regulating cell cycle (Cyclin B1 and PTTG1), transcription factor activity (OCT4), polyamine biosynthetic (ODC1), signal transduction and activation of transcription (STAT3) .

Quantitative real-time PCR results showed a higher expression of all the selected genes in good compared to poor grade oocytes except of STAT3 which showed a similar expression in camels' oocytes.

Keywords: Buffalo, camel, oocyte quality, gene expression

INTRODUCTION

Early embryonic development is supported by maternal mRNAs as well as synthesized and stored proteins in the cytoplasm of oogenesis (Rodman and Bachvarova, 1976). Maternal factors are critical for the development post fertilization and the maternal–embryonic transition, when transcription of the embryonic genome becomes fully activated. Recent studies have revealed that genes expressed in oocytes and early embryos are conserved among species, since numerous orthologs have been found in mice and humans by in silico analysis (Stanton and Green, 2001 & 2002). However, oocyte transcriptome and identity of key oocyte-expressed genes are still not fully understood.

Characterization of gene expression in oocytes will provide additional insight into the regulation of oocyte maturation, fertilization, and pre-implantation development. Oocyte quality is one of the main determinants of *in vitro* embryo production success, while culture conditions during *in vitro* embryo production are the key factor determining oocyte development to the blastocyst (Lonergan *et al.,* 2001 & 2003).

Selection of the quality oocytes for *in vitro* fertilization is assumed to be based on quality markers extracted from morphological criteria and biochemical parameters. As far as no molecular biomarker for oocyte quality has been established, thus, understanding the molecular mechanisms in oocytes is quite important for both reproductive biology and regenerative medicine (Hamatani *et al.,* 2008).

Different approaches have been applied for analyzing oocyte with different qualities based on gene expression analysis (Wang and Sun, 2007; Pandey *et al.,* 2010 and Prasad *et al.,* 2010) as more precise and objective tools. Up to our knowledge, the only work discussing this issue in buffalo was reported by Prasad *et al.* (2010) and only one report has discussed the expressed sequence tags for *Camelus dromedarius* in different adult tissues (Al-Swailem *et al*., 2010). In addition the mRNA transcript abundance of oocytes or embryos in camel has not yet been analyzed.

The present study was aimed at identifying the differences in selected transcripts (Cyclin B1, OCT4, PTTG1, STAT3 and ODC1) abundance of genes related to oocyte quality of buffalo and camel oocytes, which could be associated with oocyte developmental potential.

MATERIALS AND METHODS

Oocyte collection and evaluation in vitro

Ovaries were collected from a local abattoir, transported to the laboratory within 1-2 h in physiological saline solution at 30-35 °C and washed once in 70% ethanol and twice with warm (37°C) phosphate buffer saline (PBS) contained 50 ug/ml gentamicin. Cumulus-oocyte complexes (COCs)

were recovered by slicing ovaries (Hamano and Kuwayama, 1993). The COCs were evaluated based on their morphological features. Oocytes have 3-5 layers of cumulus cells and homogenous granular cytoplasm were considered as a good quality, while those with less than three layers or denuded were considered as poor ones (Gordon, 2003). A total of 360 COCs of buffalo and camel (n=180 each) were selected of which 90 were pooled in three replicates with 30 oocytes in each of the good and poor oocytes to be used for gene expression analysis.

Cumulus cells separation

The surrounding cumulus cells were separated from immature oocytes by gentle pipetting in washing medium supplemented with hyaluronidase 1mg/ml. Cumulus free oocytes were washed twice in PBS and snap frozen separately in cryo-tubes containing 20 µl of Lysis buffer [0.8 % IGEPAL, 40 U/µl RNasin (Promega Madison WI, USA), 5 mM dithiothreitol (DTT) (Promega Madison WI, USA)]. Finally, samples were stored at liquid nitrogen at -196°C until RNA isolation.

RNA isolation

Total RNA was isolated using PicoPure™ RNA isolation kit (MDS Analytical Technologies GmbH, Ismaning, Germany) according to manufacturer's instructions. Oocytes in a Lysis buffer were mixed with 100 µl extraction buffer and incubated at 42 ˚C for 30 min to obtain complete Lysis of the samples and to release RNA. The extract was loaded onto a pre-conditioned purification column and centrifuged to allow the RNA to bind to the spin column. DNA digestion in the column was carried out using RNase-free DNase (Qiagen GmbH, Hilden, Germany). The column was washed twice with washing buffer and finally eluted with 12 µl RNase free water. For each sample, cDNA synthesis was performed using oligo (dT) 23 primer, random primer and superscript reverse transcriptase II (Invitrogen, Karlsruhe, Germany) in addition to one micro-litre of oligo (dT) 23 primer and one micro-litre random primer were added to 10µl mRNA sample and the mixture was incubated for 3 min at 70˚C and then immediately chilled on ice. Eight micro-litres of the master mix containing 4 µl of 5x first strand buffer, 2 µl of 0.1 M DTT, 1 µl of dNTP (10 pmol/µl) and 0.3 µl of RNase inhibitor and 0.7 µl of SuperScript IITM reverse transcriptase (200 unit/µl) were added to the mixture and incubated for 90 min at 42˚C followed by heat inactivation for 15 min at 70˚C. The synthesized cDNA was stored at -20˚C for further use.

Table 1. Details of the primers used for quantitative real-time PCR.

Gene sympol	Gene bank accession number	Primer sequences	Annealing temperature (˚C)	Product size (bp)
PTTG1	NM_004219	F: 5´- GAAGAGCACCAGATTGCGC -3´ R: 5´- GTCACAGCAAACAGGTGGCA -3	55	204
OCT4	NM_174580	F: 5´- GATATACCCAGGCCGATGTG-3´ R: 5´- CTCGGTTCTCGATACTCGTC-3	53	240
Cyclin B	L48205	F: 5′ -GAGGGGATCCAAACCTTTGTAGTGA-3′ R: 5'-CAATTTCTGGAGGGTACATTTCTTC-3'	55	318
ODC1	NM_174130	F: 5´- CAAAGGCCAAGTTGGTTTTAC-3´ F: 5´-CAGAGATGGCCTGCACAAAG-3´	55	201
STAT3	XM_001494674	F: 5´-CTGTACAGTGACAGCTTCCC-3´ F: 5´-GCAATCTCCATTGGCTTCTC-3´	52	245
GAPDH	NM_001034034	F: 5´-ACCCAGAAGACTGTGGATGG-3´ R: 5´-ACGCCTGCTTCACCACCTTC-3´	55	247

Quantitative real-time PCR analysis

Real-time PCR primers were designed based on the cDNA sequences of the five transcripts available in GenBank (Table 1) using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA). Quantitative analyses of cDNA samples from independent oocytes were performed in comparison with the bovine GAPDH gene (endogenous control), and were run in separate wells using Mx3000 instrument (Stratagene).

Independent qRT-PCR (3 replicates for each group for each genotype) was performed in a 20-µl reaction volume containing iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Munich, Germany), the cDNA samples and the specific forward and reverse primer. The thermal cycling parameters were set at 95°C for 3 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. After the end of the last cycle, a dissociation curve was generated by starting the fluorescence acquisition at 60°C and

taking measurements every 7-second interval until the temperature reached 95°C. The comparative cycle threshold (CT) method was used to quantify the expression levels as previously described by Bermejo-Alvarez *et al.* (2010).

Expressions of five different transcripts (Cyclin B1, OCT4, PTTG1, STAT3 and ODC1) were analyzed using real-time PCR and fold changes were calculated using ΔΔCt method.

RESULTS

Gene expression profiling of immature oocytes with different grades

Buffalo (*Bubalus bubalis*)

Results of quantitative real-time PCR showed higher expression of all the selected genes (n=5) in good compared to poor grade oocytes (Figure 1). The highest ratio of change was recorded in Cyclin B1 and OCT4, where they showed over-expression in good grade by (2.5 fold change) followed by STAT3 (2 folds) and PTTG1 (1.6 folds), while the lowest change rate was recorded for ODC1 (1.2 folds).

Camel (*Camelus dromedarius*)

Results showed higher expression of all the selected genes in good compared to denuded oocytes except STAT3, which showed a similar expression in both good and poor oocytes**.** The higher expression was observed in OTC4 (1:3.4 folds) followed by PTTG1 (1:2.9 folds) and Cyclin B1 (1:2.4 folds). The lower expression was recorded by ODC1 (1:1.9 folds) (Figure 2).

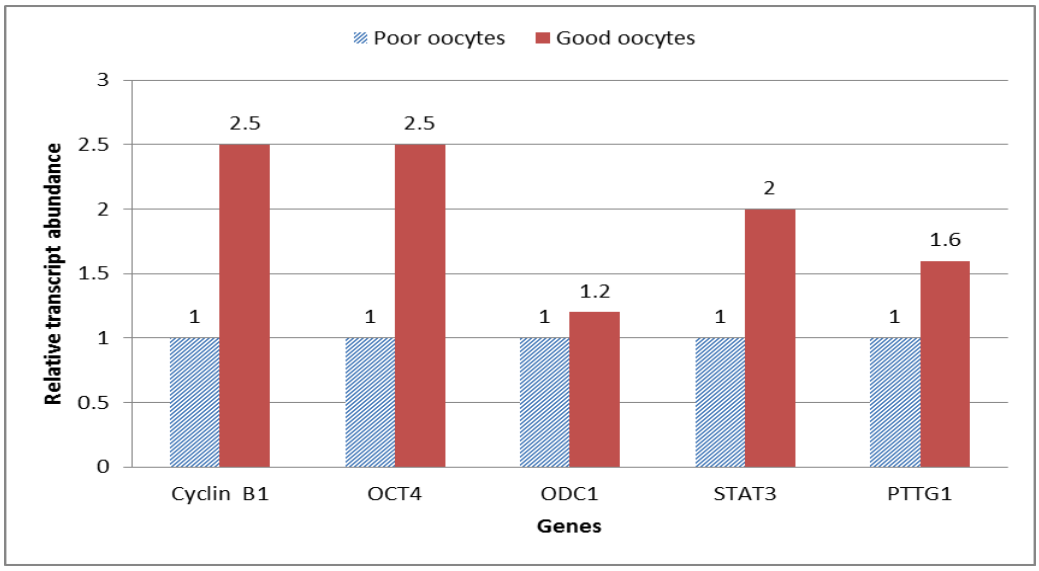

Figure 1. Differentially expressed transcript abundance of candidate genes for immature buffalo oocytes of good and poor quality

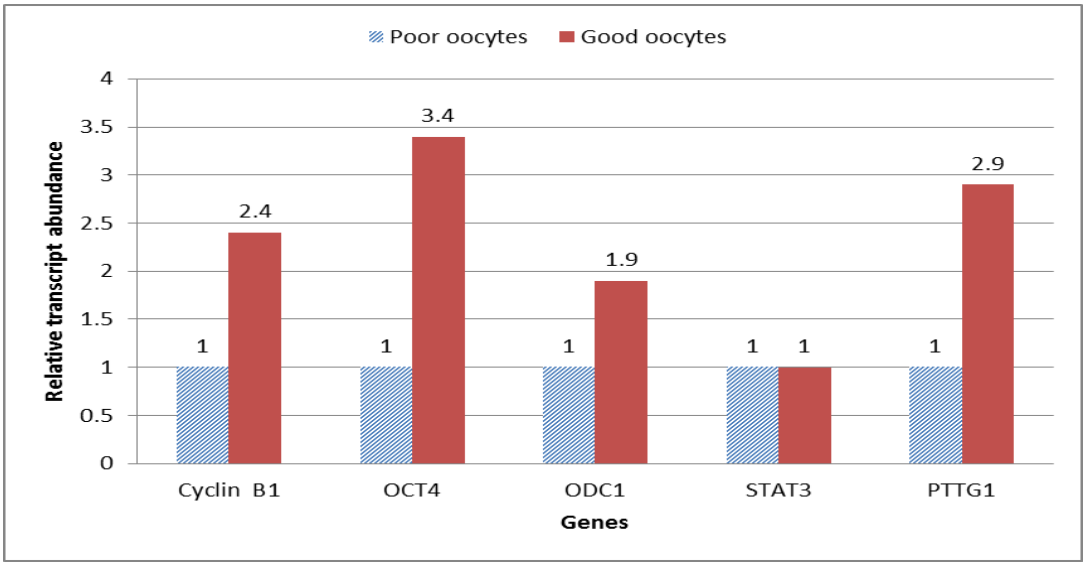

Figure 2. Differentially expressed transcript abundance of candidate genes for immature camel oocytes of good and poor quality

DISCUSSION

Molecular characteristics of oocytes are important for the selection of oocytes of developmental capacity (Patel *et al.,* 2007). Poor developmental capacity of bovine oocytes is assumed to be highly complicated and reliant on many small changes in the RNA levels of many genes (Donnison and Pfeffer, 2004).

The present results of Cyclin B1 in good and poor quality oocytes of buffaloes and camels are in agreement with the result of Hue *et al.* (1997) who reported that both Cyclin B1 mRNA and protein have been detected in meiotically competent and incompetent goat oocytes. The incompetent oocytes have a limited amount of Cyclin B1. Low expression of Cyclin B1 in poor quality oocytes reflects the importance of this gene for both quality and maturation of oocytes.

The obtained expression ratio of OCT-4 in good oocytes by more than 2 and 3 times compared to poor counterparts in both buffaloes and camels respectively is in agreement with the result obtained by Donnison and Pfeffer (2004). The authors reported 1.2 fold increase in OCT-4 transcript in oocytes derived from large follicles (> 5 mm) relative to those derived from small ones (< 2 mm). Moreover, Prasad *et al.* (2010) stated that, A and B grade oocytes showed strong OCT-4 expression. Naked oocytes showed faint or no detectable signal and as a result OCT-4 may be one of those specific genes whose expression is dependent on oocyte quality, suggesting that the lower developmental potential of naked oocytes might be due to the absence of OCT-4 transcripts.

ODC1 gene was up-regulated in good grade oocytes by 1.2 fold change compared to poor ones in buffaloes increased to 1.9 in camels' good grade oocytes. The present results reflect the importance of this transcript for the development and maturation. These results are confirmed by Paula-Lopes *et al.* (2007) and Torner *et al.* (2008). They reported an over-expression of this gene in competent bovine immature oocytes compared to incompetent counterparts. In addition, Mohammadi-Sangcheshmeh *et al.* (2011) and Smits *et al.* (2011) found the same trend in equine oocytes.

STAT3 showed an increase in buffalo good grade oocytes by two times fold change compared to poor ones. This result is in agreement with the previous results on bovine obtained by Torner *et al.* (2008) and Mohammadi-Sangcheshmeh *et al.* (2011) who studied the expression level of this gene in equine oocytes. However, this gene showed similar expression in both good and poor grade camel oocytes. The opposite trend for this gene in camel compared to bovine and buffaloes need more studies at a large scale and using different models of oocyte selection. A lot of precautions should be paid when using different species as a model for camel *in vitro* embryo production application.

The relative abundance for PTTG1 was higher in good grade oocytes by 1.6 times in buffalo and 2.9 times in good grade compared to poor ones in camel oocytes. The present result is in contrary with the result obtained by Ghanem *et al.* (2007). In addition, PTTG1 gene was found to be up-regulated in embryo biopsies derived from blastocysts, which resulted in no pregnancy after transfer to recipients (El-Sayed *et al.,* 2006). On the other side, this result supports the importance of this gene for further development as down-regulation of PTTG1 delays the resumption of meiosis and over-expression of PTTG1 permits the resumption of meiosis even in the presence of inhibitory concentrations of cAMP (Marangos and Carroll, 2008). Expression level for both cyclin B1 and PTTG1, in the present study, it could be speculated a relation between these two genes which is proofed by Marangos and Carroll (2008) who found that the depletion of PTTG1 increased CyclinB1 degradation, resulting in delayed progression into M-Phase of cell cycle during mouse oocyte maturation (Marangos and Carroll, 2008).

Collectively from the expression patterns of the selected genes for this study, it could be concluded that, the reported differences in developmental competence of buffalo and camel oocytes derived from different quality are also accompanied by differences in the relative abundance of transcripts related to the various molecular events and processes regulating oocyte competence. Our validation with an independent model for the relative abundance of some selected transcripts, supports the notion that many of the transcripts identified as differentially regulated and described here may represent marker candidate genes for oocyte developmental competence. However, the exact role of these transcripts in controlling oocyte developmental potential needs further investigation.

To our knowledge, only one report has discussed one gene related to oocyte quality in buffalo (Prasad *et al.,* 2010) and one report studied the expressed sequence tags for *Camelus dromedarius* in different adult tissues (Al-Swailem *et al.,* 2010). The mRNA transcript abundance of oocytes in camel has not yet been analyzed. The present results have added new insights into IVF in camels at the molecular level. Many more studies are needed to explore the difference between this species and other species like bovine as it's the golden standard for farm animals at this level of research.

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مقارنة التعبيير الجينى للجينات ذات العلاقة بجودة البويضة فى كلٍ من بويضات الجاموس و الجمال المنتجة معمليا

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تعتبر جودة البويضة واحدة من المحددات الرئيسية للنجاح في إنتاج الاجنة معمليا. من اجل ذلك تم استخدام نهج مختلفة لتحليل صفات جودة البويضة و بصورة خاصة تحليل التعبير الجيني، الذي يمثل أداة جديدة لاختيار البويضات الجيدة. تهدف الدراسة الحالية إلى دراسة التعبير الجيني للبويضات غير الناضجة ذات الصفات الشكلية الجيدة و غير الجيدة فى كلا من الجاموس والجمال. تمت الدراسة على عدد 360 بويضة من الجاموس والجمال (ن = 180 لكل منهما) . فى كل نوع كان يتم تجميع 90 بويضة لكل فئة (جيدة و غير جيدة) في ثلاثة مكررات تحتوى على 30 بويضة استخدمت لتحليل التعبير الجيني بعدإزالة الخلايا الحبيبية من حولها. تم استخلاص RNA من جميع العينات باستخدام PicoPure^{TM} ثم تم تحويله الى cDNA عن طريق النسخ العكسي. بعد ذلك تم عمل Real-Time PCR باستخدام بادئات محددة لمجموعة من الجينات ذات الوظائف المختلفة مثل تنظيم دورة الخلية (Cyclin B1 and PTTG1)، نشاط عامل النسخ (OCT4)، التخليق الحيوى لعديد الأمين (ODC1)، نقل الإشارات وتنشيط عملية النسخ (STAT3). أظهرت نتائج Real-Time PCR زيادة التعبير الجينى لجميع الجينات المختارة فى البويضات الجيدة مقارنة بغير الجيدة فيما عدا STAT3 الذى لم يتغير التعبير الجينى له فى بويضات الجمال.