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# EXPRESSION OF SELECTED CANDIDATE GENES DURING *IN VITRO* OOCYTE MATURATION AND EARLY EMBRYONIC DEVELOPMENT OF *IN VITRO* GENERATED EGYPTIAN BUFFALO (*BUBALUS BUBALIS*) EMBRYOS

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## SUMMARY

*The present study aimed at finding out the gene expression analysis of some candidate genes during in vitro maturation of Egyptian buffalo oocytes and in early embryonic developmental stages. Good quality oocytes were recovered from ovaries of slaughtered buffalo cows subjecting to in vitro maturation and fertilization process. Real-time PCR was used for expression analysis of genes regulating cell cycle (PTTG1), calcium ion (ANAX2), prostaglandin biosynthesis (PTGS2), protein binding (KRT8) and translation (EEF1A1) during target stages.*

*Quantitative real-time PCR results showed a high expression of all the selected genes in mature oocytes except PTTG1 and KRT8 that showed a lower expression after maturation. The transcript abundance of PTGS2, EEF1A1, KRT8 and ANAX2 genes increased slightly at 4- to 8-cell stage embryos and continued to increase till blastocyst stage. PTTG1 expression profile was decreased at 4- to 8-cell stage embryos up to blastocyst stage.*

*In conclusion, selected candidate genes showed a spatiotemporal expression during in vitro maturation and early embryonic development which reflects that transcription regulation of early development in buffaloes is also stage dependent as other species but has some peculiarities when compared to bovine.*

***Keywords: Buffalo, oocyte, embryo, gene expression***

## INTRODUCTION

Oocyte maturation is an essential step in *in vitro* embryo production (IVEP) system. IVEP has been introduced to buffaloes to increase the number of offspring from elite females, and to reduce the generation intervals (Suresh *et al.*, 2009). Despite the progress made in IVEP in buffalo for producing morula /blastocyst and establishing pregnancy (Madan *et al.*, 1994a & b) number of transferable embryos (TE) and those developed to term is still very low (Madan *et al.*, 1996) and less than anticipated.

Attempts to improve IVEP in buffaloes is in progress via understanding the molecular mechanisms of buffaloes' oocytes maturation based on expression analysis of candidate genes using Real-Time PCR (Pandey *et al.*, 2009 & 2010 and Jain *et al.*, 2012).

Oocytes store vital information in the form of proteins and messenger RNA of various genes during growth process, therefore the optimal storage of these molecules and the spatiotemporally availability during oocyte maturation are crucial to early embryo development (Gandolfi 1998; Brevini-Gandolfi *et al.*, 1999; Brevini *et al.*, 2002 and 2006). Changes or alteration of gene expression during oocyte maturation could be deleterious for further embryonic development (Su *et al.*, 2007). In addition, a relative abundance of selected candidate mRNAs could be used as marker genes to check the developmental competence of mature oocytes (Pandey *et al.*, 2010). Therefore, five candidate genes from our previous studies (El-Sayed *et al.*, 2006, Ghanem *et al.*, 2007 and Torner *et al.*, 2008) were selected to be analyzed during different stages (immature, mature oocytes, 2-cell, 4-cell, 8-cell, morulla and blastocyst) of buffalo embryos generated *in vitro*.

The aim of the present study was to analyze gene expression profile of buffalo oocytes during *in vitro* maturation and selected stages of pre-implantation embryos using Real-time PCR for specific candidate genes.

## MATERIALS AND METHODS

### *Chemicals and media:*

Unless otherwise mentioned, all the chemicals, reagents, media, biologics and media constituents were purchased from Sigma-Aldrich Chemicals, Germany. The final media for maturation and fertilization were filtered (0.22 µm poor size, Durapure® membrane filter, Ireland) and routinely equilibrated at 39 °C with 5% $CO_2$ in humidified air for at least two h before using it. Only cell culture tested chemicals were used to formulate the media.

### *Oocyte collection and in vitro maturation:*

Ovaries of Egyptian buffalo (n= 634) were collected from a local slaughterhouse and

transported in normal saline solution at 35 ˚C to the lab within one to two h. Cumulus oocyte complexes (COCs) were aspirated from 3-8 mm follicles using an 18 gauge needle fixed to five ml syringe. The COCs were morphologically selected based on the number of cumulus cell layers and cytoplasm homogeneity (Furuns *et al.,* 1998 and Gordon 2003). Oocytes enclosed with three to five layers of cumulus cells and homogenous granular cytoplasm were considered as a good quality and were utilized for *in vitro* maturation and gene expression analysis.

A total of 550 COCs were selected of which 50 were pooled in five replicates (n= 10 each). Oocytes were kept as immature for gene expression analysis after cumulus cells denudation. The rest 500 COCs were underwent *in vitro* maturation for three independent runs (each with about 166 oocytes) for 24 h in tissue culture medium [TCM-199 with HEPES, L-glutamine, 50 µg/ml Gentamycin, 10 µg/ml FSH and 10% fetal calf serum (FCS, Sigma, USA)] at 39 ˚C under 5% $CO_2$ in humidified air. Oocyte maturation was evaluated based on cumulus expansion and extrusion of 1$^{st}$ polar body.

***In vitro fertilization and embryo culture:***

Frozen semen from Egyptian buffalo bulls was used for *in vitro* fertilization. Three straws of frozen semen were thawed at 37°C for 30 seconds and sperms were washed twice using centrifuge at 500 g for 10 min. with Sperm Tyrod's Albumin Lactate Pyruvate medium (Sperm-TALP) containing 10 µg/ml heparin, 2.2 mg/ml sodium pyruvate and BSA F-V (6 mg/ml) + 50 µg/ml Gentamycin. After washing, a sperm pellet was suspended in 0.5 ml of fresh Fert-TALP medium supplemented with six mg/ml BSA (fatty acid free) + 10 µg/ml heparin + three µl PHE and 50 µg/ml Gentamycin. Sperm concentration was adjusted to 2 x $10^6$ spermatozoa/ml. A total of 300 µl of motile sperm suspension was placed in 4-well culture plate and covered with mineral oil. *In vitro* mature oocytes were washed in Fert-TALP media three times and placed into the sperm suspension (40 oocytes / well) and kept at 39 °C under 5% $CO_2$ in humidified air for 18-20 h. The fertilized oocytes were thoroughly washed in a modified synthetic oviductal fluid (mSOFaa) supplemented with five mg/ml BSA + five ng/ml insulin and 50 µg/ml Gentamycin. The presumptive zygotes (10-15 zygotes/50 µl droplets and covered with mineral oil) were cultured in mSOFaa medium at 39°C under 5% $CO_2$ in humidified air.

The stage of embryonic development was evaluated daily till day seven, and the media was replaced with fresh one every 48 h.

***Cumulus cells separation:***

The surrounding cumulus cells were separated from immature and mature oocytes by gentle pipetting in maturation medium supplemented with hyaluronidase one mg/ml. Separation of cumulus cells was carefully checked under a stereomicroscope. 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), five mM dithiothreitol (DTT) (Promega Madison WI, USA)]. Finally, samples were stored at liquid nitrogen at -196°C until RNA isolation.

***RNA isolation:***

Oocyte RNA extraction was performed at different time points during the whole experiment. Five pools each with 10 oocytes or embryos were obtained from immature oocytes, mature oocytes, 2-cell, 4-cell, 8-cell, morulla and blastocyst.

Total RNA was isolated using PicoPure™ RNA isolation kit (MDS Analytical Technologies GmbH, Ismaning, Germany) according to manufacturer's instructions. Oocytes or embryos 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 for the RNA to bind to the spin column. DNA digestion in 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 µl of oligo (dT) 23 primer and one µl random primer were added to 10µ1 mRNA sample and the mixture was incubated for three min at 70˚C and then immediately chilled on ice. Eight µl of the master mix containing four µl of five x first strand buffer, two µl of 0.1 M DTT, one 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.

***Quantitative real-time PCR analysis:***

Real-time PCR primers were designed based on the cDNA sequences of the five transcript gene sequences 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 ABI PRISM® 7000 sequence detection system (Applied Biosystems). Prior to quantification, primer optimisation was performed for both forward and reverse primers. Specific primer level combinations with lower threshold cycle ($C_T$) value and without primer-dimer formation were selected for subsequent PCRs. Standard curves were generated for both target and endogenous control genes using serial dilution of plasmid DNA ($10^1 - 10^9$ molecules). The PCRs were performed in 20 µl reaction volume containing of 10 µl of 2.5 X RealMasterMix/20x SYBR (Eppendorf, Hamburg, Germany). During each PCR reaction, samples from the same cDNA source were run in duplicate to control the reproducibility of real-time results. A universal thermal cycling parameter (10 sec at 50 ˚C, 10 min at 95 ˚C, 45 cycles of 15 sec at 95 ˚C and 60 sec at 60 ˚C) was used to quantify each gene of interest. After the end of the last cycle, dissociation curve was generated by starting the fluorescence acquisition at 60 ˚C and taking measurements every seven sec interval until the temperature reached 95 ˚C. Final quantitative analysis was done using the relative standard curve method and the results were reported as the relative expression (El-Sayed *et al.,* 2006 and Ghanem *et al.,* 2007).

***Statistical analysis:***

The relative expression data were analyzed using the Statistical Analysis System (SAS) version 8.0 (SAS Institute Inc., Cary, NC, USA) software package. Differences in mean values were tested using analysis of variance followed by a multiple pairwise comparison using t-test. Differences of $P \leq 0.05$ were considered to be significant

## RESULTS

***Efficiency of IVM, IVF and IVC:***

Maturation rate of buffalo oocytes cultured in TCM199 was 76.9 % as determined by cumulus expansion and first polar body extrusion. Embryo cleavage rate was 71.81 % while percentage of zygote that reached blastocyst stage was 20.7 % (Table 2).

***Gene expression profiling of immature vs. mature buffalo oocytes:***

Expression of all genes was detected during *in vitro* maturation process. The transcript abundance of PTGS2, EEF1A1, and ANAX2 genes was up-regulated in mature compared to immature oocytes. While the expression profile of KRT8 and PTTG1 transcripts was down-regulated in mature compared to immature oocytes (Figure 1-5).

***Gene expression profiling of cleaved buffalo embryos:***

The transcript abundance for all genes was also detected throughout different stages of buffalo embryonic development. In general, the expression profile was reduced at 2, 4 and 8 cell embryo stages. On the other hand, there is abrupt increase in the expression of all genes during the development of morulla to blastocyst stage except PTTG1. The expression profile of PTTG1 gene was in descending pattern throughout early embryonic stages to reach plateau at blastocyst (Figure 4). While KRT8 showed ascending expression profile to be at maximum expression at blastocyst (Figure 5).

## DISCUSSION

***Efficiency of in vitro embryo cleave:***

Pre-implantation embryonic development and differentiation are regulated by a well-orchestrated expression of genes (Brevini *et al.,* 2006 and Adjaye *et al.,* 2007). Transcription and translation are increasing during oocytes growth leading to formation of RNAs and proteins (Fair *et al.,* 1997), which required for resumption of meiosis (Tatemoto and Horiuchi, 1995) and regulating maternal to zygotic transition (Hyttel *et al.,* 2001).

Elongation factor is a component of the eukaryotic translational apparatus and it is also a GTP-binding protein that catalyses the binding of aminoacyl tRNAs to the ribosome (Tatsuka *et al.,* 1992). The tRNA carries the amino acid to the ribosome, which is then used in protein synthesis. Thus, it is not surprising that EEF1A1 gene was up-regulated during buffalo oocyte maturation as the requirement of proteins regulating maturation process is increasing. This result is supported by findings of Kandil *et al.* (2010) that showed a higher expression of several genes associated with translation machinery in mature buffalo oocytes. Expression of this gene was reduced at 2 cell till morulla stage but abruptly increased at blastocyst stage. The lower transcription of this gene at these stages may be due to transition from maternal to embryo transition.

In the current study, the expression profile of calcium ion binding regulating gene ANXA2 was greater in mature compared to immature oocytes. This result is accordance with one of our microarray study done in buffalo oocytes (Kandil *et al.,* 2010). Up-regulation of ANXA2 was also reported in pig and mice mature oocytes compared to early stages of embryonic development in both species (Cui *et al.,* 2005). The protein profile

of ANAX2 was higher in normal mature compared to aged porcine oocytes (Jiang *et al.,* 2011). This provides evidence for the importance of this gene in determining oocyte ability to maturation and subsequent fertilization (Kandil *et al.,* 2010). On the other hand, our result showed lower expression of ANAX2 at earlier embryonic stages but increased at blastocyst. In bovine, ANAX2 transcript abundance was higher in immature oocytes and blastocyst stages (El-Halawany *et al.,* 2004). Despite similar expression of this gene in both species at blastocyst stage an opposite trend was detected in mature oocytes. This finding indicated that biological requirements of buffalo oocytes and embryos are not the same as of bovine subsequently buffaloes *in vitro* embryo production should be modified in the way that fits this species.

Prostaglandin G/H synthase-2 (PTGS2)-derived appeared to be critical mediators of the cumulus expansion and oocyte maturation processes (Hizaki *et al.,* 1999). So, higher expression of PTGS2 in mature compared to immature oocytes in our study could reflect the crucial role of this gene in regulating maturation process. PTGS2-derived PGE2 acts directly on the oocyte via PTGER2 to activate the proteins involved in meiosis progression in cattle (Nuttinck *et al.,* 2011). Our results showed a relatively stable expression throughout embryonic development. This could be due to the crucial role of this gene as a molecular determinant of timely regulation of cell cycle progression and early embryonic development (Nuttinck *et al.,* 2011).

Cytokeratin 8 (KRT8) is the early and fundamental keratin expressed together with keratin 18 during development of many vertebrates (Jackson *et al.,* 1980), and the main keratin present in hyper-proliferative human cells (Moll *et al.,* 1982). Our results observed higher expression profile of KRT8 in immature compared to mature oocytes. Furthermore, it has showed an ascending expression from 2-cell stage embryos to reach the highest expression at blastocyst stage. The lower expression of KRT8 at maturation could be due to degradation of a large proportion of stored transcripts within the oocyte cytoplasm as found in bovine (Fair *et al.,* 2007) or due to post-transcriptional regulation through shortening of the polyA tail (deadenylation) (Paynton and Bachvarova, 1994). While the up-regulation of KRT8 at blastocyst stage is explained by it is vital contribution in blastocyst formation (Goossens *et al.,* 2007).

Pituitary tumor transforming gene (PTTG1) is important for maintaining chromosome stability, cell cycle progression, and appropriate cell division (Wang *et al.,* 2001). In oocytes, this gene is required for efficient chromosome segregation (Assou *et al.,* 2006). This gene was up-regulated in mature oocytes (Chu *et al.,* 2012). However, in our data of the current study PTTG1 was decreased it expression in mature buffalo oocytes and continue to decline to reach lowest expression at blastocyst. This pattern is similar to maternal regulated transcripts (Pennetier *et al.,* 2004). This also could be due to its deadenylation process that occurred during maturation (Thélie *et al.,* 2007). Although there is reversed expression of this gene during bovine and buffalo oocytes maturation it is suggested to play a role in meiotic resumption and fertilization (Chu *et al.,* 2012). PTTG1 was found to be up-regulated in blastocysts resulting in no pregnancy compared to those resulted in calf delivery and suggested to be marker of bad quality blastocyst (El-Sayed *et al.,* 2006). However, Mourot *et al.* (2006) reported increased expression of this gene in good quality earlier cleaving bovine embryos. The elucidation of actual role of this gene in embryonic development could be done by target degradation of mRNA using RNA interference technique.

In conclusion, the data presented here has clearly shown that the expression of selected candidate genes is spatio-temporal throughout buffalo pre-implantation and influenced dramatically by maturation and transition from maternal to embryo genome activation periods. The expression of two genes (ANAX2 and PTTG1) in buffalo oocytes was reversed compared to bovine so; more care should be given when applying bovine IVP procedure in buffaloes. Further investigations are required to find out the gene expression profile of in vitro compared to in vivo produced buffalo embryos.

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

| Gene sympol | Gene bank accession number | Primer sequences | Annealing temperature (˚C) | Product size (bp) |
|---|---|---|---|---|
| ANXA2 | NM_174716 | F: 5´- CGTGCTCCAGCTAACAGTTCT-3´<br>R: 5´- GGAAAGCCAGGTAATGCGTA-3 | 55 | 139 |
| PTTG1 | NM_004219 | F: 5´- GAAGAGCACCAGATTGCGC -3´<br>R: 5´- GTCACAGCAAACAGGTGGCA -3 | 55 | 204 |
| EEF1A1 | AB060107 | F: 5´- CCATGGCATATTAGCACTTGGTT-3´<br>R: 5´- GCTTACACCCTGGGTGTGA-3´ | 55 | 214 |
| PTGS2 | NM_174445.2 | F: 5´- GGAAATCCTCAGCTCAAAAC-3´<br>R: 5´- GTCTCCCTGGGAACTATTC-3´ | 60 | 221 |
| KRT8 | X12877 | F: 5´-: CACCAGTTCCAAGCCTGTGG-3´<br>R: 5´- TCAGGTCTCCTGTGCAGATGC-3´ | 54 | 176 |
| GAPDH | BC102589 | F: 5´-ACCCAGAAGACTGTGGATGG-3´<br>R: 5´-ACGCCTGCTTCACCACCTTC-3´ | 60 | 247 |

**Table 2.** Maturation and development rates (%) of *in vitro* produced buffalo embryos

| Stage<br>No and % | Collected oocytes | Mature oocytes | Fertilized oocytes | Cleaved embryo | Blastocyst/cleaved |
|---|---|---|---|---|---|
| Oocyte No. | 450 | 346 | 330 | 237 | 49 |
| Percent (%) | | 76.9± 3.3 | | 71.8 ± 2.0 | 20.7 ± 2.9 |

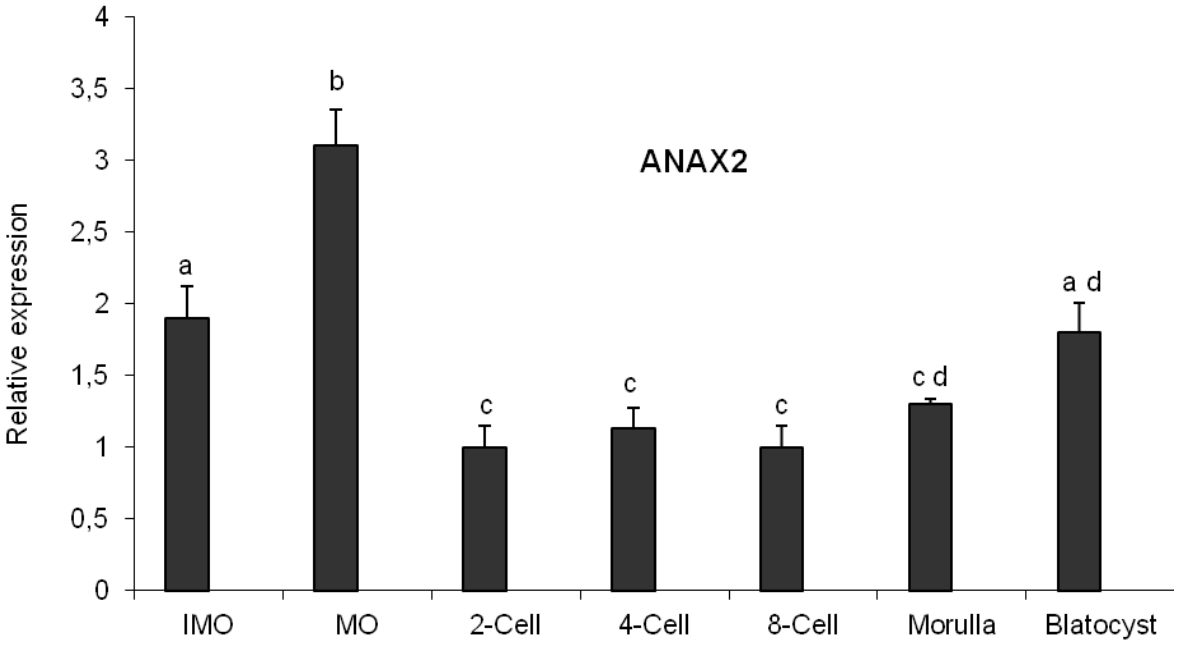


**Figure 1. Expression profile of ANAX2 gene throughout Egyptian buffalo pre-implantation using Quantitative real-time PCR. Bars with different superscripts (a, b, c, d) are significantly different at P<0.05**

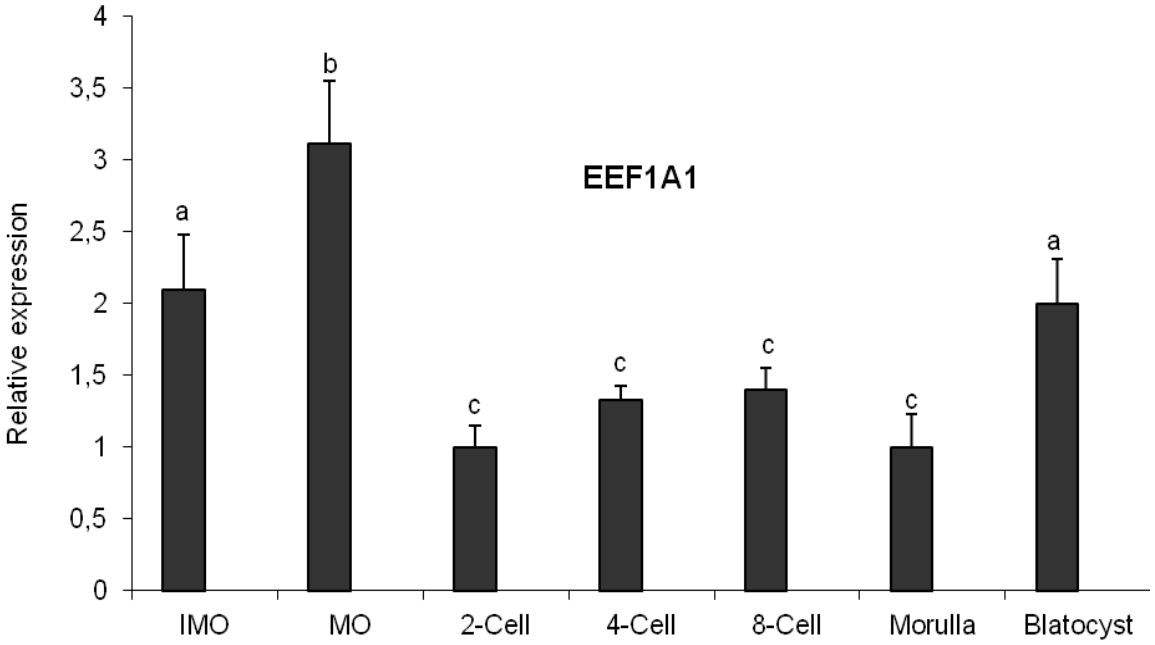


**Figure 2. Expression profile of EEF1A1 gene throughout Egyptian buffalo pre-implantation using Quantitative real-time PCR. Bars with different superscripts (a, b, c, d) are significantly different at P<0.05**

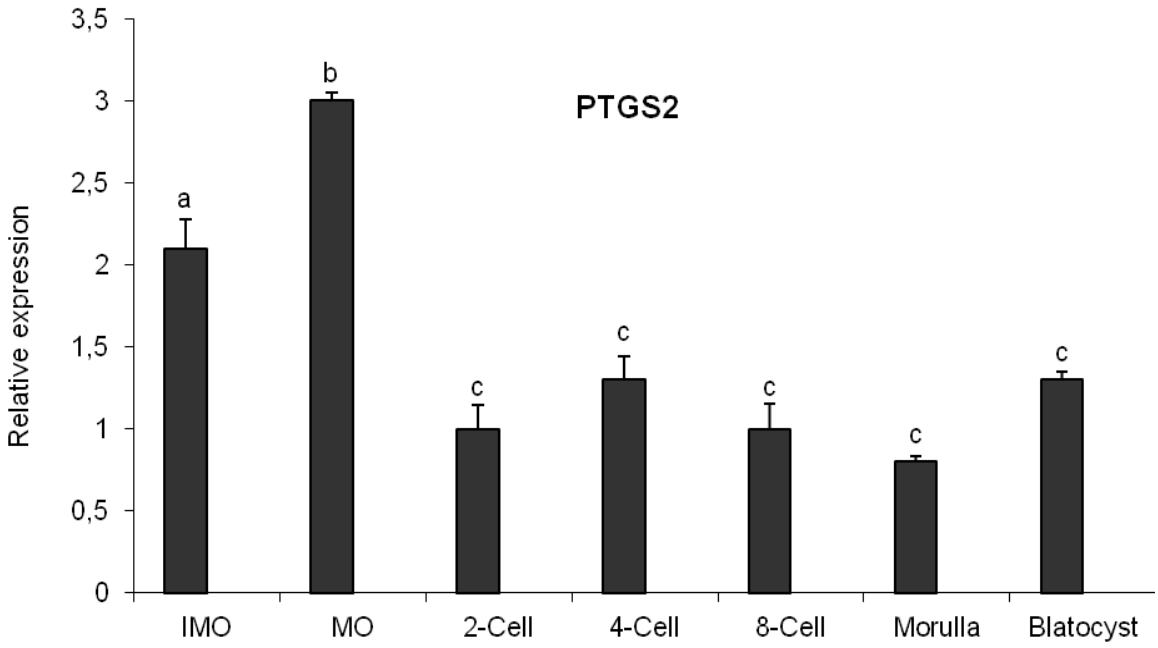


**Figure 3. Expression profile of PTGS2 gene throughout Egyptian buffalo pre-implantation using Quantitative real-time PCR. Bars with different superscripts (a, b, c, d) are significantly different at P<0.05**

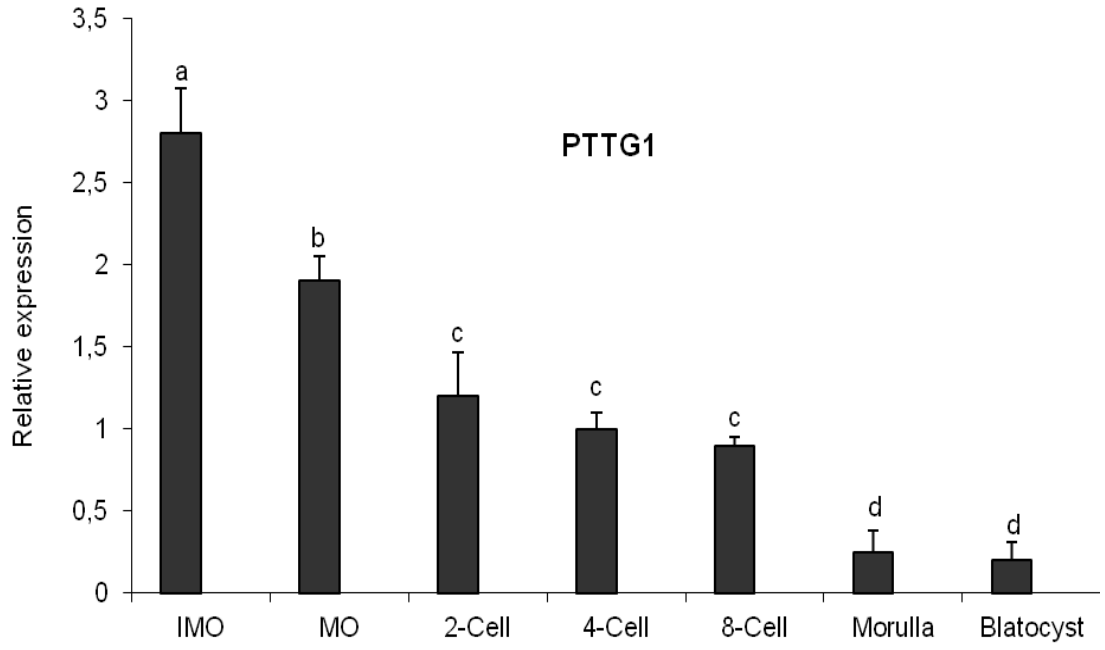


**Figure 4. Expression profile of PTTG1 gene throughout Egyptian buffalo pre-implantation using Quantitative real-time PCR. Bars with different superscripts (a, b, c, d) are significantly different at P<0.05**

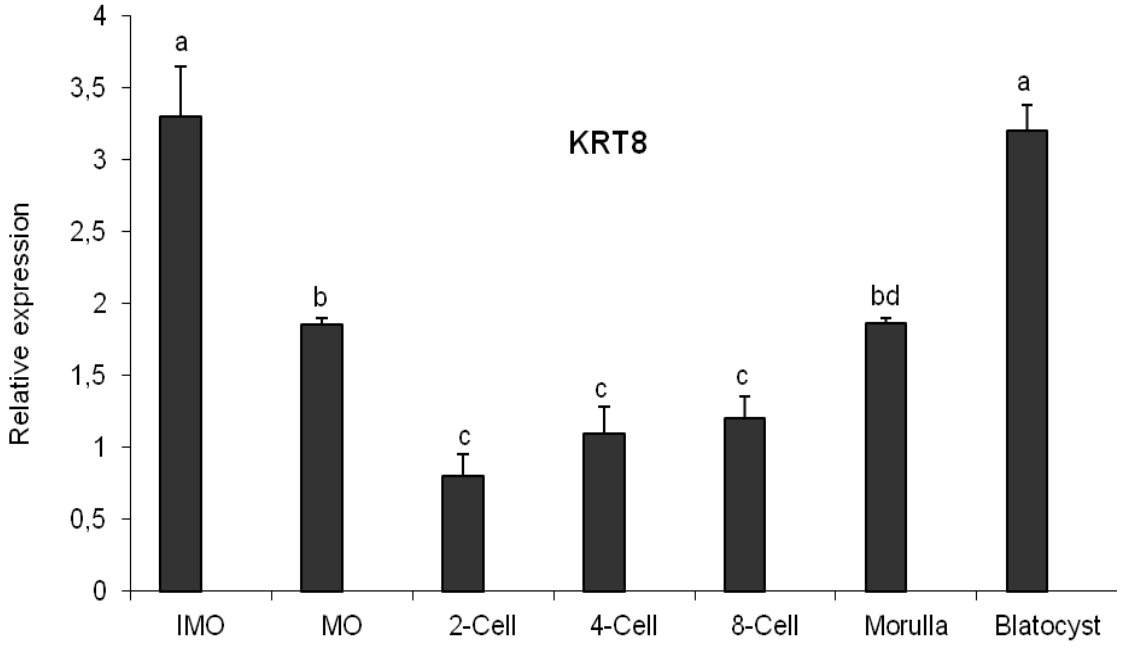


**Figure 5. Expression profile of KRT8 gene throughout Egyptian buffalo pre-implantation using Quantitative real-time PCR. Bars with different superscripts (a, b, c, d) are significantly different at P<0.05**

# التعبير الجينى لعدد من الجينات فى كلا من بويضات و اجنة الجاموس المصرى المنتجة معملياً

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تعتبر عملية الانضاج المعملى للبويضات من اهم الخطوات لانجاح عملية انتاج الاجنة معمليا للحيوانات المزرعية بصفة عامة. لذا تهدف هذه الدراسة الى اكتشاف و تحليل التعبير الجينى لبعض الجينات المميزه اثناء عملية الانضاج المعملى لبويضات الجاموس المصرى و كذلك اثناء النمو الجنينى فى مرحلة ما قبل الانغراس. لهذا الغرض تم جمع البويضات عالية الجوده من مبايض الجاموس المصرى المذبوحة فى احدى المجازر المحلية بناء على التقييم المورفولوجى ثم تم انضاجها معمليا و اخصابها و زراعتها فى بيئات نمو لاستكمال النمو و التطور حتى مرحلة البلاستوسيست.بعد ذلك تم دراسة التعبير الجينى لبعض الجينات التى لها علاقة بانضاج البويضات و كذلك المراحل الاولى من النمو الجنينى باستخدام تقنية تفاعل البلمرة المتزامن Real time PCR . بعض هذه الجينات لها علاقة بدورة الخلية مثل PTTG او تنظيم ايونات الكالسيوم ANAX2 و التخليق الحيوى لهرمون البروستجلاندين PTGs2 و عملية الارتباط بالبروتينات KRT8 و عملية الترجمة داخل الخلية EEF1A1 .

أظهرت نتائج الدراسة, زيادة التعبير الجينى لثلاث من الجينات بعد الانضاج المعملى و هم ANAX2 و PTGs2 و EEF1A1 فى حين انخفض كلا من PTTG و KRT8 . اما فى مرحلة النمو الجنينى نجد ان التعبيير الجينى قد زاد لكل من ANAX2 و PTGs2 و EEF1A1 و KRT8 بشكل بسيط عند مرحلة 4 خلايا الى 8 خلايا و استمر فى الزيادة التدريجية حتى مرحلة البلاستوسيست. نستخلص من هذه الدراسة ان التعبير الجينى لبعض الجينات يختلف فى البويضات الناضجة عن البويضات قبل الانضاج و كذلك فى المراحل المختلفة من النمو الجنينى قبل عملية الانغراس, مما يعنى ان عملية نسخ الجينات اثناء النمو الجنينى فى الجاموس تختلف باختلاف مرحلة النمو و التطور كما فى بعض الحيوانات المزرعية الاخرى مثل الابقار.