RESEARCH ARTICLE Indian Journal of Animal Research, Volume 58 Issue 11: 1854-1859 (November 2024)

# Comparison of Gene Expression for Preimplantation Genes in Bovine Embryos Fertilized *in vitro*

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10.18805/IJAR.BF-1838

## ABSTRACT

**Background:** *In vitro* fertilization (IVF) the technique is useful for understanding early mammalian development and maximizing and speeding up genetic improvement in livestock. Genes that function as genetic markers and are involved in the pre-implantation development of embryos may be used to evaluate the quality of an embryo and the expression of these genes is correlated with the timing of embryonic genomic activation.

**Methods:** Maturing the oocytes of bovine, then fertilizing these matured oocytes and finally culturing the fertilized oocytes to develop into embryos. Determined the gene expression of different certain genes HSP70, OCT4, GLUT1, BAX and DNMT1 in these different cell stages of embryos that were produced.

**Result:** The mRNA expression of these genes was estimated after collecting the embryos listed above that were produced by the *in vitro* technique. The results showed statistically significant differences in the relative abundance between some of the genes mentioned above in each embryo stage. The timing of the zygotic genome activation process (ZGA) can result in differences in gene expression levels between the early embryonic genes in each developmental stage of *in vitro*-produced bovine embryos.

**Key words:** Embryo development, Gene expression in bovine, *In vitro* culture, Maturation in bovine.

## INTRODUCTION

Animal production uses biotechnology to increase the variety of livestock animal species, supply the world with animal products, protect endangered species to increase reproduction, conserve genetic diversity and biodiversity to provide goods and services for the welfare of the human population and support economic progress. *In vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), somatic cell nuclear transfer (SCNT), semen and oocyte cryopreservation, embryo cryopreservation and embryo sexing, embryo transfer, stem cell and other assisted reproductive procedures (ARTs) are included in this biotechnology (Abdullah *et al*., 2005; Andrabi and Maxwell, 2007). *In vitro* maturation of oocytes followed by *in vitro* fertilization (IVF) and then the *in vitro* culture are the steps to complete the full experiment for the *in vitro* fertilization technique.

During the preimplantation development stages, mammalian embryos are exposed to several alterations in their gene expression. After fertilization, the zygotic genome activation process begins with the breakdown of maternal transcripts during the conversion of an oocyte into an early embryo and the initiation of new transcription called zygotic genome activation (ZGA) (Wang *et al*., 2005), so the study of gene expression during preimplantation embryonic development is critical for understanding the changes that occur during the transition from gametes to pluripotent embryos and into the early stages of cell differentiation and the quantity of RNA transcripts has been documented extensively for various genes in bovine oocytes and preimplantation embryos (Ross *et al*., 2010).

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**How to cite this article:** Al-Malahi, N.M., Daghestani, M.H., Al-Ghadi, M.Q., Alkhuriji, A.F., Amran, R.A. and Alhimaidi, A.R. (2024). Comparison of Gene Expression for Preimplantation Genes in Bovine Embryos Fertilized *in vitro*. Indian Journal of Animal Research. 58(11): 1854-1859. doi: 10.18805/IJAR.BF-1838.

**Submitted:** 30-06-2024 **Accepted:** 10-08-2024 **Online:** 16-09-2024

Gene expression represents the events collected during embryo development from post-fertilization to the stage of the blastocyst, this includes the formation of the zygote, the first stages of cleavage, zygotic genome activation, compacted morula and blastocyst formation resulting in a particular level of mRNA in a cell, so the extraction of mRNAs from cell populations is used to assess gene expression profiles for various genes, if the ZGA is absent, the developing embryo will die because it cannot able to support the essential developmental functions (Larson *et al*., 2009; Sagirkaya *et al*., 2006).

The genes chosen in our study have an important role in embryonic development, during embryonic development the maintenance of the homeostasis in the cells is important, some agents can regulate the homeostasis between cell death (apoptosis) and prevent continuous cell death (anti-apoptosis); the agents that regulate apoptosis belong to the Bcl-2 family. Changes that cause

cell death, include a loss of cell volume, DNA fragmentation, membrane blebbing, protein cleavage and protein cross-linking, which ends with the formation and release of apoptotic bodies containing the cellular components of the dying cell which are then phagocytized by neighboring cells, so the expression for the BAX gene (apoptotic gene) is important during these changes, in contrast, the BCL2 gene (anti-apoptotic gene) will prevent the continuous death of cells to maintains the hemostasis in the embryos to complete the development (Aliparasti *et al*., 2015; Aqeilan *et al*., 2003; Li *et al*., 2009; Matwee *et al*., 2000).

Tolerance in cells also can be protected by the gene HSP70 which is named the cytoprotective factor that protects the cells from cellular stress or transfection which stimulates reactive oxygen species (ROS) that leads to a variety of cellular damage, such as disrupting the embryo cytoskeleton, intracellular calcium levels which have been associated to the impairment of cellular functions, the mitochondrial function (de Barros and Paula-Lopes, 2018; Luft and Dix, 1999). DNA methylation is an epigenetic mechanism that plays an important role in early preimplantation embryo development in mammals (Shi *et al*., 2023; Zhang *et al*., 2023); which is achieved by adding a methyl group to the fifth carbon atom of cytosine, this mechanism is vital for maintaining genome stability in cells, DNMT1 gene is responsible for maintaining this normal methylation pattern (Chen and Zhang, 2020; Urbanek-Olejnik *et al*., 2014). OCT4 gene (Octamer-binding transcription factor-4) belongs to the POU transcription factor family; it is a transcription factor that is important in differentiation and also demanded to maintain the inner cell mass pluripotency (Cherepanova *et al*., 2016; Nordhoff *et al*., 2001; Pan *et al*., 2002). Glucose is an important energy substrate for the development of bovine embryos and GLUT1 is one of the Glucose transporters proteins family (GLUTs) that employ the diffusion of glucose gradient across the cell plasma membrane as a primary source of energy and as a metabolic intermediate (Ostrowska *et al*., 2015) and after that metabolized through the glycolytic pathway or the phosphate pathway (Harvey *et al*., 2002; Thompson *et al*., 1996).

## MATERIALS AND METHODS

### Oocyte collection and maturation

The *in vitro* fertilization experiment was achieved after aspirating the Cumulus oocyte complexes (COCs) from the bovine ovaries collected from the slaughter. These COCs were matured in TCM-199 Earle's salt maturation medium for 21-24 h at 39°C in an atmosphere of 5% $CO_2$ in the air with maximum humidity as already stated in these study (Al-Malahi *et al*., 2022; Ammari *et al*., 2022; Amran *et al*., 2024).

### Sperm capacitation

The cryopreserved semen of the bull was acquired from the Center of Asset Conservation and Genetic Enhancement located in Al-Kharj City, associated with the Saudi Ministry of Environment, Water and Agriculture and prepared according to (Al-Malahi *et al*., 2022; Amran *et al*., 2023).

### *In vitro* fertilization

After COCs maturation and sperm capacitation, 10-15 COCs were cultured in drops of 50 μl each per 60 mm Petri dish containing *in vitro* fertilization medium (IVF-BO) supplemented with 25 μg/ml gentamycin, 11.12 μg/mL heparin, 1.25 mM pyruvate and 3 mg/ml bovine serum albumin under mineral oil. Finally, capacitated motile sperm were added to the COCs with the mentioned final concentration above, for 24 h incubation in a humidified atmosphere of 5% $CO_2$ at 39°C (Al-Mutary *et al*., 2019).

### *In vitro* culture

*In vitro* culture medium (IVC-SOF) (Caisson IVL05) supplemented with 1 mM L-glutamine, 0.34 mM sodium pyruvate, 50X MEM-essential amino acids, 100X MEM nonessential amino acids (Sigma M7145), 25 μg/ml gentamycin, 3 mg/mL BSA, 1.5 mM glucose and 1 μg/ml EDTA in a 35 mm dish, used to culture the fertilized and

**Table 1:** Primers for the studied genes that were employed in real-time polymerase chain reaction amplification.

| Gene | Function | Primer sequences (5-3) Forward (F) and Reverse (R) | Gen bank accession no. |
|---|---|---|---|
| GAPDH | Reference gene | F: GGTTGTCTCCTGCGACTTCAA<br>R: AATGCCAGCCCCAGCAT | NM_001034034.1 (Cánepa *et al*., 2014) |
| HSP70 | Stress | F: AGCAAAGAACCAAGTCGCAATG<br>R: AAGGTAGGCTTCTGCGATTTCC | BC105182 (Rodríguez-Alvarez *et al*., 2010) |
| OCT4 | Pluripotency | F: GGTTCTCTTTGGAAAGGTGTTC<br>R: ACACTCGGACCACGTCTTTC | NM174580 (Rodríguez-Alvarez *et al*., 2010) |
| GLUT1 | Glucose transporter | F: CTGATCCTGGGTCGCTTCA<br>R: GGATACCTCCCCCACGTACA | M60448 (Amarnath *et al*., 2007) |
| BAX | Apoptosis | F: GCGCATCGGAGATGAATTG<br>R: CCAGTTGAAGTTGCCGTCAGA | U92569 (Amarnath *et al*., 2007) |
| DNMT1 | DNA methylation | F: TTCGGAACTTCGTCTCCTTCA<br>R: GCCAAAGGTGCACTGGTACC | AY173048 (Amarnath *et al*., 2007) |

unfertilized COCs after denuded by using the hyaluronidase enzyme solution. Zygotes were cultured in a droplet of the mentioned medium in an incubator at 39°C in a 5% $O_2$, 5% $CO_2$ and 90% $N_2$ for 7-9 days with high humidity (Alotaibi *et al*., 2022).

**Embryo collection and freezing**

Bovine embryos were collected during the 7-9 days of culture in different cleavage stages for the stages from the 2-cell stage, to the blastocyst stage. After being cleaned in 0.1% PVA, these embryos were frozen in the same solution and kept at -80°C until the RNA extraction (Al-Malahi *et al*., 2022; Duarte De Oliveira *et al*., 2005).

**RNA extraction for gene expression**

The protocol steps for RNA extraction, cDNA synthesis and gene expression analysis, to determine the relative abundance of the following genes in this study: HSP70 (heat shock protein), OCT4 (pluripotency gene), GLUT1 (glucose transporter), BAX (an apoptosis gene) and DNMT1 (DNA

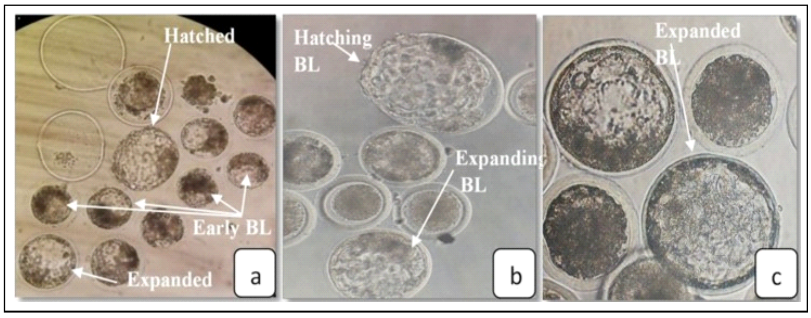


**Fig 1:** Different stages of blastocysts (BL).

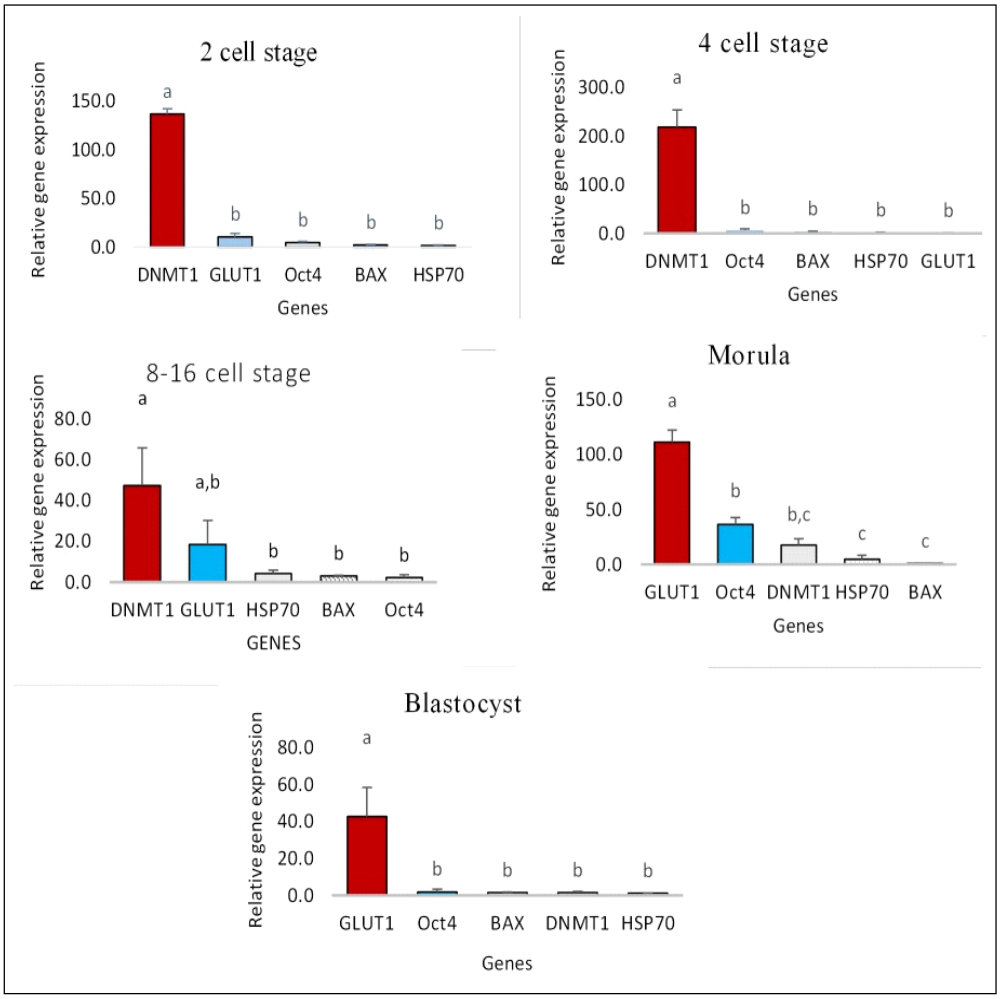


**Fig 2:** Relative gene expression of all genes investigated at each stage of embryonic development.

methyltransferase), have been detailed in our prior research study (Al-Malahi *et al*., 2022). The forward and reverse primers used in this work are listed in Table 1.

The gene expression for the target studied genes was measured by using the Comparative Ct ($2^{\Delta\Delta Ct}$) method and was normalized to the reference GAPDH gene (Housekeeping Gene) as the following (Amarnath *et al*., 2007):

Step 1: Calculate the average of the duplicate Ct values for the GAPDH gene and the target studied genes.
Step 2: ($\Delta$Ct = target gene Ct - housekeeping gene Ct).
Step 3: Calculate the average of the $\Delta$Ct values.
Step 4: ($\Delta\Delta$Ct = sample $\Delta$Ct - average highest sample $\Delta$Ct).
Step 5 and the last: Calculate the $2^{\Delta\Delta Ct}$.

The work was achieved in the Zoology Department, King Saud University, Riyad, Saudi Arabia for around 10 months.

**Statistical analysis**

Software called SPSS 20 was used to do the statistical analysis. One-way ANOVA was used to examine mean differences and then multiple pairwise comparisons using Duncan's test were conducted to investigate relative differential gene expression (IVP) in bovine embryos. The data were given as the mean±standard error. P-values were significant when they were less than 0.05 (EM *et al*., 2014).

## RESULTS AND DISCUSSION

A total of 974 oocytes were cultured to study the cleavage stages of the bovine embryos and the different embryo blastocyst stages (early, expanding, expanded, hatched and hatching blastocysts) that are shown in (Fig 1) and the rate of embryo blastocyst was studied in embryos that fertilized *in vitro*. The rate of the blastocyst stage was calculated by the following equation:

Blastocyst rate (30%) =

$$\frac{\text{No. of blastocyst embryos (66)}}{\text{No. of cleaved embryos (217)}} \times 100$$

The level of gene expression of all studied genes HSP70, OCT4, GLUT1, BAX and DNMT1 of *in vitro* fertilization bovine embryos in each cleavage stage including the 2-cell, 4-cell, 8-16 cell, morula and blastocyst stages collected on different days during the 7-9 days of culture. Our results showed an increase in the gene expression of the DNMT1 gene compared to the rest genes in the 2-cell stage and this height difference is significant, the same result significant increase was cleared for the same gene DNMT1 compared to the rest genes in the 4-cell stage. There was a height difference that is significant in the gene expression of the DNMT1 gene compared to the genes HSP70, BAX and OCT4 but not significant with the GLUT1 gene in the 8-16 cell stage. As for the morula stage, there was a significant increase in the gene expression of the GLUT1 gene compared to the rest genes and a significant increase in the expression of the OCT4 gene compared to HSP70 and BAX genes, but not significant with the DNMT1 gene, while in blastocyst stage there was a significant increase in the gene expression of the GLUT1 gene compared to the rest genes (Fig 2).

Gene expression returns to the collection of events that result in a specific level of mRNA in a cell, so the extraction of mRNAs from cell populations is used to evaluate gene expression profiles for different genes (Larson *et al*., 2009), our study illustrated the differences between the gene expression of the genes HSP70, OCT4, GLUT1, DNMT1 and BAX in different cell stages (2 cells, 4 cell, 8-16 cell, morula and blastocyst stages) in embryos produced *in vitro,* which considered the genes that are used as genetic markers and play roles in the pre-and post-implantation development of embryos (EM *et al*., 2014).

Our results showed that gene expression of the DNMT1 gene in 2 cell stage had significantly high regulation compared to the other genes, this return to the high storage of mRNA for DNMT1 in oocytes before the embryonic genome activation causes the DNMT1 mRNA to remain in very high levels and starts to low after that (Duan *et al*., 2019), the same result appeared in the 4-cell stage. According to Urbanek-Olejnik *et al*. (2014) that showed during cell division most genes are inactive and DNMT1 is from the genes that are responsible for regulating the gene expression for these genes, which is important for embryo development.

DNMT1 showed decreasing expression in 8-16 cells, which agreed with (Dor and Cedar, 2018) who illustrated that the methylation level decreased after the 8-cell stage. GLUT1 showed increased expression in the same embryo stage but was non-significant with the other genes, but this increase was consistent with (Lopes *et al*., 2007) who reported that expression of this gene is low during the first cleavages and increases sharply after the embryonic genome activation so the glucose metabolism increasing to give more energy for the embryo to complete the development.

The expression of the GLUT1 gene showed a significant increase in the morula stage compared to the other genes and this is consistent with (Lopes *et al*., 2007) who cleared that expression for this gene increases during compaction formation because the embryo glucose uptake increases to help for development and this would show the vitality of the embryo and the decreasing in expression of the other genes, which may indicate that the embryo is not exposed to any stress. As for the OCT4 gene increased compared to the other, this result was similar to the findings (Kurosaka *et al*., 2004) which indicated that expression for this gene starts to increase after embryonic genome activation. Low expression for the other genes may explain why the embryos were not exposed to more stressors needing high regulation for these genes.

The expression of this gene in the blastocyst stage was significantly higher than the other genes, the high regulation for this gene agreed with the study (Mucci *et al*., 2006) illustrated that the embryo consumes more glucose

with the blastocyst formation and expansion and is also consistent with (Wrenzycki *et al*., 2003) who reported the same information. Downregulation for the other genes may return to hemostasis during embryo development.

## CONCLUSION

According to the results mentioned the gene expression of the studied genes in preimplantation embryos produced *in vitro* differs from each other due to the stage embryo which determines the timing of expression of these genes during this stage, this timing is called the zygotic genome activation which begins to activate with the breakdown of the maternal genomic, however, that some genes have high expression before this timing which that returns to the high storage for it in the oocytes.

## ACKNOWLEDGEMENT

The authors gratefully applaud the Researcher Support Project (RSP 2024/97) at King Saud University in Riyadh, Saudi Arabia, for financing this work. Our gratitude and admiration also go to the Central Riyadh slaughterhouse for their assistance in acquiring bovine ovary samples.

### Conflict of interest

The authors declare no conflict of interest.

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