BF-1724
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RESEARCH ARTICLE Indian Journal of Animal Research

Impact of Ionomycin on the Gene Expression of *In vitro* Fertilized Bovine Embryos

R.A. Amran[1], A.R. Alhimaidi[1], A.A. Ammari[1], M.G. Al Ghadi[1], N.M. Al-Malahi[1]

10.18805/IJAR.BF-1724

ABSTRACT

Background: The embryo genomic material used in this study came from an equal contribution of maternal and paternal genes. Gene expression during IVEP embryo development was controlled by several factors: the genetic makeup of the embryo, the epigenetic contributions and the culture materials. The aims of this research were to study the impact of ionomycin treatment on the sperm capacitation time and its impacts on the gene expression of some sensitive genes (Bcl-2, Bax, HSPB1, IGF1 and GDF9) during IVEP.

Methods: The semen was placed in a sterile 15ml tube containing 5ml of capacitation medium and then split into three different experimental groups: the first group was the control; the second was the low-treated group, which contained sperm in a capacitation medium with 25 nM of ionomycin; the third group included 50 nM ionomycin.

Result: The gene expression of BAX during early cleavage did not show any differences. However, in the late cleavage stage, the control group presented higher gene expression ($P<0.05$) than that in the two treated groups. The Bcl-2 genes of the control group showed higher gene expression ($P<0.05$) in both the early and late cleavage stages compared to the low-treated group. Gene expression of the HSP-1 gene was observed during early embryo development in the treated group (25 nM ionomycin). In conclusion, concentration 25 nM ionomycin showed higher gene expression in the early stages of embryo development also the best-quality embryos and gene expression results were found by studying gene expression. The transition from the maternal and paternal genome to the embryonic genome and gene expression during embryo development is controlled by several factors.

Key words: Bovine embryos, Gene expression, *In vitro* fertilized, Ionomycin.

INTRODUCTION

Modern reproductive biotechnology involves harvesting of oocytes *via* aspiration, *in vitro* maturation (IVM), fertilization and *in vitro* cultures (IVCs). Within the same duration of time, IVEP can generate two to three times as many embryos as multiple ovulation embryo transfer (MOET). According to the International Embryo Transfer Society (IETS) 2016 report on domestic animal embryo collection and transfer, since oocytes can be removed using the aspiration of ovaries obtained from slaughterhouses and ovum removal technologies from live animals, the IVEP process is more flexible than MOET (Binyameen *et al.,* 2019).

In vitro embryo production (IVEP) is an important technique in cow breeding that has experienced a significant increase in utilization. Throughout the world, commercial companies produce bovine embryos (Camargo *et al.,* 2006; Abd El-Aziz *et al.,* 2016; Stoecklein *et al.,* 2021; Blaschka *et al.,* 2021).

The generation of male and female pronuclei and extrusion of the second polar body are characteristics of the second stage of *in vitro* production, also referred to as *in vitro* fertilization (Parrish, 2014). The presence of spermatozoa with a capacitated acrosome reaction in close proximity to mature oocytes is required for *in vitro* fertilization to initiate. Such sperm have the ability to penetrate the oocyte's zona pellucida and combine with the ooplasm during fertilization (Stival *et al.,* 2016). Changes in cholesterol level, lipid composition, calcium permeability, fluidity and membrane surface properties are thought to cause capacitation (Davis, 1981). Most of these alterations are connected to modifications in the spermatozoa's plasma membrane, supporting the theory that capacitation is a process of membrane maturation (Jones, 1997; Sukardi *et al.,* 2001).

The capacitation of spermatozoa *in vitro* is accomplished by washing the sperm in a medium containing equivalent amounts of electrolytes, metabolic energy sources and substances that affect the spermatozoa's viability and motility in addition to the induction and speed of the acrosome reaction process.

Ionomycin is a calcium ionophore that induces an acrosome reaction by increasing Ca^{2+} cycling across the phospholipid membranes (Ball *et al.,* 1983). The calcium ionophore A23187 artificially triggers the acrosome reaction in the spermatozoa of many species, including horses

[1]Department of Zoology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia.

Corresponding Author: A.A. Ammari, Department of Zoology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia. Email: aammari@ksu.edu.sa

How to cite this article: Amran, R.A., Alhimaidi, A.R., Ammari, A.A., Al Ghadi, M.G. and Al-Malahi, N.M. (2024). Impact of Ionomycin on the Gene Expression of *In vitro* Fertilized Bovine Embryos. Indian Journal of Animal Research. DOI:10.18805/IJAR.BF-1724

Submitted: 27-10-2023 **Accepted:** 14-01-2024 **Online:13-02-2024**

(Varner *et al.,* 1987; Zhang *et al.,* 1991; Magistrini and Palmer, 1990; Farlin *et al.,* 1992). Ca^{2+} controls sperm hyperactivated motility and fertilization in mammalian sperm species. Sperm's ability to fertilize changes when the Ca^{2+} channel CatSper is genetically lost (Clapham, 2007). On the other hand, infertility occurs in mice without the plasma membrane Ca^{2+} ATPase (PMCA4b) due to a lack of progressive and hyperactivated motility (Okunade *et al.,* 2004). Proteins that depend on Ca^{2+} interact with Ca^{2+} as a second messenger. Ca^{2+} either directly or indirectly activates many effectors in the sperm (Clapham, 2007). The most frequently reported effector is Calmodulin (CaM), which exhibits a considerable suppression of tyrosine phosphorylation when inhibited with an antagonist. Sperm motility, but not viability, was negatively impacted by CaM inhibitors (Zeng and Tulsiani, 2003). By controlling and stimulating the activities of numerous enzymes, including Ca^{2+}/CaM-dependent protein kinases, CaM has been proven to perform other functions. These enzymes include Phosphodiesterase 1, which hydrolyzes cAMP during sperm capacitation and eventually regulates protein kinase A in the sperm and Calcineurin (Baxendale and Fraser, 2005). A crucial part of Ca^{2+} signaling is performed by calcineurin, a Ca^{2+} or calmodulin-dependent serine/threonine phosphatase (Klee and Wang, 1998).

The final stage of *in vitro* production (IVP) is the *in vitro* culturing of bovine embryos, which begins with a zygote culture and normally takes 7 to 8 days. At this stage, the embryo undergoes its first cleavage division, the embryonic genome is activated and the morula and blastocyst are created (Wrenzycki, 2018; Ramos-Deus *et al.,* 2020; Nogueira *et al.,* 2021).

The culture conditions have the ability to change gene expression not only in the oocyte stage but also in the blastocyst stage (Lonergan *et al.,* 2003; Nemcova *et al.,* 2006; Rizos *et al.,* 2002).

For *in vitro* embryo production to be optimized, an understanding of gene expression during oocyte maturation is essential (Aswal *et al.,* 2008). Oocytes may express apoptotic genes (such as Bcl-2, an antiapoptotic gene and Bax, a pro-apoptotic gene) under certain *in vitro* maturation conditions. The ratio of pro- and anti-apoptotic genes may also affect the sensitivity of cells to apoptotic stimuli (Ebrahimi *et al.,* 2010; Kim and Tilly, 2004; Yang and Rajamahendran, 2002).

Bax gene expression was shown to be higher in degenerated embryos compared to that in healthy embryos Yang *et al.* (1998) and when serum was added to the culture media (Rizos *et al.,* 2002; Sanna, 2009). Heat stress, freezing and minor pH changes all caused the heat shock protein (HSPB1) to be produced (Chernik *et al.,* 2004). This protein has a significant impact on a number of biological processes, including gene activation, cell cycle arrest, differentiation and apoptosis (Palasz *et al.,* 2008). These genes (Bax, Bcl-2 and HSPB1) could be considered good indicators of oocyte maturity.

In sheep, GDF-9 and BMP-15 are essential for normal follicle growth and oocyte production (Dube *et al.,* 1998; Li *et al.,* 2008).

This study aims to evaluate the expression of certain genes (Bcl-2, Bax, HSPB1, IGF1 and GDF9) in different stages of bovine embryos produced *in vitro* by investigating the effects of applying 25 and 50 nM ionomycin to the sperm capacitation medium.

MATERIALS AND METHODS

Chemicals and reagents

Sigma provided all of the chemicals and media for this experiment and computations (St Louis, Missouri, United States) unless otherwise specified.

Ionomycin solution

A 1 mg bottle of calcium ionomycin salt (Sigma I0634) was thawed using a DMSO 134 µl solution to make a 10 mM stock solution and then stored at -20°C until use.

Frozen semen

The Saudi Ministry of Environment, Water and Agriculture's Center for Asset Conservation and Genetic Improvement in Al-Kharj City provided us with frozen sperm from Holstein–Friesian cattle.

Collection of oocytes and *in vitro* maturation

Within two hours, ovaries were collected from the central slaughterhouse in Riyadh, Saudi Arabia and transported to the lab in 0.9% sodium chloride (NaCl). The follicles were classified as small (S: 3 mm), medium (M: 3-6 mm), or large (L: >6-10 mm) based on their diameter. Aspiration was utilized in order to remove the oocytes from each of the three distinct types of follicles. TCM199 handling media (Hank's salts) with 50 g/ml gentamicin sulfate, 10% Fetal Bovine Serum (FBS) and 0.5 mM sodium pyruvate was injected using a sterile syringe. Oocytes with three or more layers of intact cumulus cells and homogenous cytoplasm were obtained using a mouth pipette and a stereomicroscope. Next the cumulus-oocyte complexes underwent three washings in 100 µl of TCM-199 maturation medium (Earle's salts) (Sigma M4530; Merck KGaA, Darmstadt, Germany) with 0.02 IU/ml follicle-stimulating hormone (FSH) (Sigma F8174), 0.023 IU/ml luteinizing hormone (LH) (Sigma L5269), 1 µg/ml Estradiol (E2) (Sigma E2758), 10% FBS, 50 µg/ml gentamicin sulfate and 0.5 mM Na-Pyruvate. After being separated into ten groups, the cumulus–oocyte complexes were placed in 50 ml maturation drops in 60 mm Petri dishes, coated with mineral oil and incubated at 39°C in a humidified atmosphere of 5% CO_2 for 24 hours (Ammari *et al.,* 2022a; Ammari *et al.,* 2022b).

In vitro fertilization of oocytes

A Bracket-Oliphant medium was used to incubate the sperm; this medium contained 4 mM potassium chloride, 112 mM sodium chloride, 0.5 mM magnesium chloride hexahydrate,

2.25 mM calcium chloride dihydrate, 0.83 mM sodium dihydrogen phosphate dehydrate, 0.05 mM phenol red and 37 mM sodium bicarbonate. The medium also contained 1.25 mM sodium pyruvate. Sperm straws were thawed for 30 seconds in 37°C water after being frozen. After thawing, a discontinuous Percoll gradient was used (Sigma GE17-0891-01) (45 and 90% (vol/vol) Percoll) (Maziero *et al.,* 2020; Nogueira *et al.,* 2021). Semen was inserted in a 15 mL tube with 5 ml capacitation medium. The pooled semen was centrifuged with the capacitation medium before fertilization at 1800 rpm for 5 minutes after being washed twice to remove any ionomycin residues from the capacitation medium. The resulting pellet was diluted to a concentration of 2×10^6 sperm per ml and then divided into four groups for testing. There were three different treatments: the control, in which the sperm were capacitated in water alone; the DMSO group, in which the capacitation medium was supplemented with DMSO solution; and the low-treated group, in which the capacitation medium was supplemented with ionomycin at a concentration of 25 nM. The highest treatment dose was given to the last group, the capacitation medium supplemented with 50 nM ionomycin. The oocytes were four times washed in the preceding capacitation medium (BO-IVF) before transferring them to the fertilization drops. The fertilization medium with 10 oocytes per 50 µl drop was inseminated with 2×10^6 sperm and incubated at 39°C in a humidified environment with 5% CO_2 for 24 hours (Amran *et al*., 2023).

In vitro culture of embryos

After 24 hours of fertilization, the cumulus cells were completely removed from the zygotes by continuously pipetting them in a hyaluronidase enzyme solution using a glass Pasteur pipette (Thermo Fisher,15202699). Next, we rinsed the zygotes in an *in vitro* culture medium (IVC-SOF) (Caisson IVL05) containing 0.34 mM sodium pyruvate, 1 mM L-glutamine, 1% (v/v) MEM-essential amino acids (50X) (Sigma B6766), 1% (v/v) MEM nonessential amino acids (100X) (Sigma M7145), 3 mg/mL BSA (Sigma A6003), 25 µg/ml gentamicin, 1.5 mM glucose and 1 µg/ml EDTA. The zygotes were cultured in a 35 mm Petri plate (BD Falcon, 351008) with 50 µl drops of the SOF medium and 25 embryos in each drop, followed by the addition of mineral oil (Sigma M 5310). Embryos were incubated for 7 to 8 days at 39°C with 5% CO_2, 90% N2 and 5% O_2 in a highly humid atmosphere (Ammari *et al.,* 2022a; Ammari *et al.,* 2022b; Amran *et al.*, 2023).

RNA isolation and reverse transcription and Real-time polymerase chain reaction

A PureLinkTM RNA mini kit (Cat. no. 12183-018A, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was utilized to isolate RNA from the embryos. Then, we added 0.4 ml of lysis buffer to each sample, followed by vortexing, The RNA was purified in accordance with the manufacturer's recommendations. Using a high-capacity complementary DNA reverse transcription kit (Cat. no. 4368814, Thermo Fisher Scientific), cDNA was synthesized with 10 µl RNA, 2 µl 10XRT buffer, 2 µl random primers, 0.8 µl dNTP mix (100 mM), 1 µl multiscribe reverse transcriptase and 4.2 µl nuclease-free H_2O mixed in a 200 µl PCR tube. Then, the samples were placed in a thermocycler according to the following program: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The synthesized cDNA was stored at -20°C prior to real-time PCR. Amplification with SYBR green master mix (Thermo Fisher Scientific) was performed on an Applied Biosystems ViiA 7 real-time PCR system (Thermo Fisher Scientific) in a 12.5 µl reaction to assess the gene expression of BAX, BCL-2, HSPB1, GDF9 and IGF1 relative to that of the housekeeping gene GAPDH. Forward and reverse primers and the approximate sizes of the amplified fragment of all transcripts are listed in Table 1. In clear 96-well plates containing multiple samples, all genes of interest were analyzed in duplicate. Amplification was carried out in a 12.5 µl reaction mixture containing 6.25 µl of SYBR Green, 0.25 µl of each forward and reverse primer, 2 µl of the cDNA template and 3.75 µl nuclease-free water. The RT-PCR program was as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 seconds; annealing at 60°C for 1 min and extension at 95°C for 15 seconds and a final extension at 60°C for 1 min (Al-Malahi *et al.,* 2022).

Table 1: Primers used for real-time PCR.

Functions	Gene	Primer sequence (5-3) Forward and Reverse	Fragment size (bp)	Gene Bank accession no. and references
Reference gene	GAPDH	F: GGTTGTCTCCTGCGACTTCAA	64	NM_001034034.1
		R: AATGCCAGCCCCAGCAT		Cánepa *et al.* (2014).
Apoptosis	Bax	F: GCGCATCGGAGATGAATTG	130	U92569
		R: CCAGTTGAAGTTGCCGTCAGA		Amarnath *et al.* (2007).
	Bcl-2	F: GCCGAGATGTCCAGTCAGC	150	
		R: GACGCTCTCCACACACATGAC		Germoush *et al.* (2018).
Stress	HSPB1	F: TCCCTGGACGTCAACCACTTCG	391	NM174068.1
		R: AGGTTTGGCGGGTGAGGATGTC		Germoush *et al.* (2018).
Granulosa cell development	GDF9	F: TCGGACATCGGTATGGCTCT	86	NM_174681.2
		R: GGATGGTCTTGGCACTGAGG		Boruszewska *et al.* (2014).
Cell development and differentiation	IGF1	F: TTGCACTTCAGAAGCAATGG	209	NM_001009774.3
		R: ACTGGAGAGCATCCACCAAC		De Brun *et al.* (2015).

Statistical analysis

Statistical analysis was performed with the Mine Tab INSTAT program. The means of relative gene expression in all groups were compared by one-way analysis of variance (ANOVA) and post-LSD Dunnett's test. Three replicates of gene expression of all genes were used.

RESULTS AND DISCUSSION

Gene expression in the early stages of embryogenesis

Impact of ionomycin on the expression levels of genes related to apoptosis (Bax and Bcl-2)

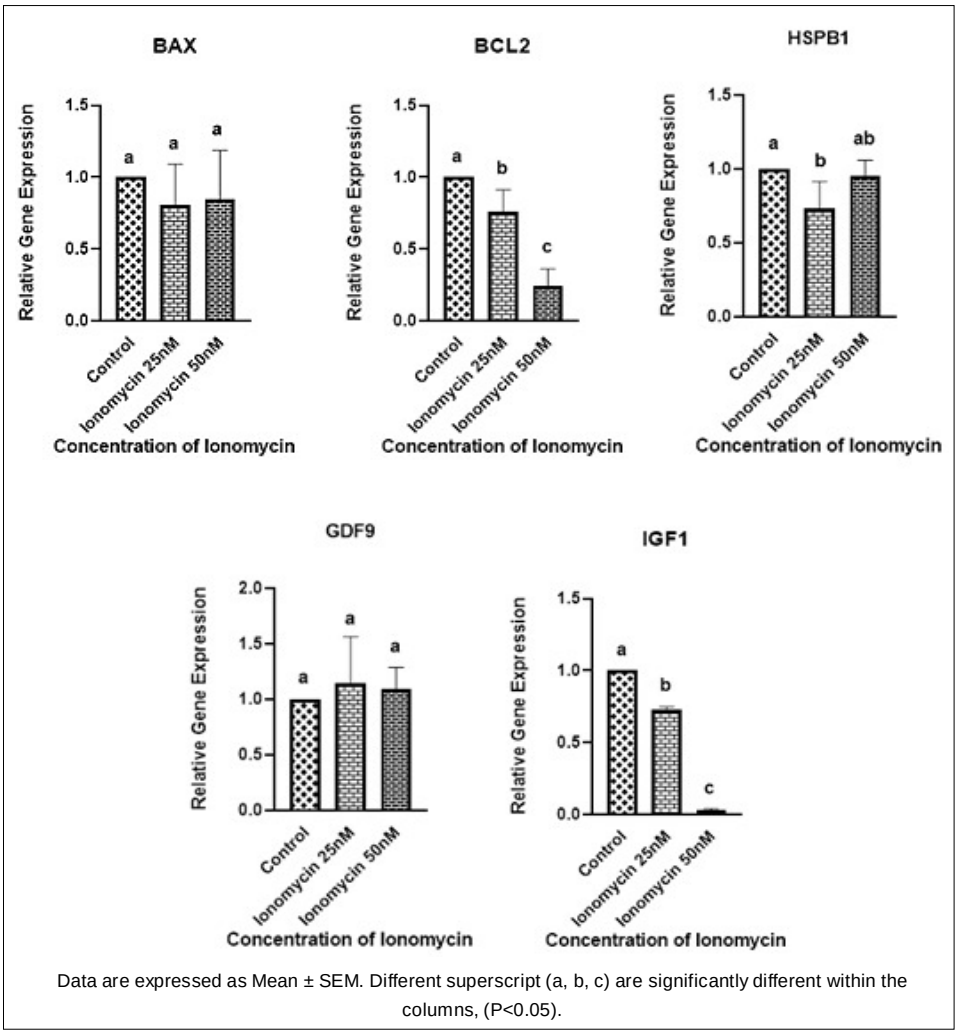
Gene expression of the BAX gene was estimated in embryos from the early stages of embryogenesis. We observed lower gene expression of the BAX gene in the groups supplemented with 25 and 50 nM ionomycin compared to that in the control group. However, the difference was not significant between the groups. In the group given 50 nM ionomycin, gene expression of the BCL2 gene was significantly ($P<0.05$) reduced compared to that in the control and Ionomycin 25 nM groups (Fig 1).

Impact of ionomycin on the expression levels of genes related to stress (HSPB1)

The effect of ionomycin supplementation on HSPB1 gene expression in embryos from the early stages of embryogenesis was studied. Compared to the control group, there were significant differences ($P<0.05$) in the gene expression of the HSPB1 gene in the group supplemented with 25 nM ionomycin (Fig 1).

Impact of ionomycin on the expression levels of genes related to granulosa cell development, nuclear maturation and embryo development (GDF9)

Gene expression of the GDF9 gene was estimated in embryos starting from the early stages of embryogenesis. Compared to the control group, we observed higher gene expression of the GDF9 gene in the groups supplemented



Data are expressed as Mean ± SEM. Different superscript (a, b, c) are significantly different within the columns, ($P<0.05$).

Fig 1: Impact of ionomycin on BAX, BCL-2, HSBP1, GDF9 and IGF1 gene expression ratio in bovine embryos from the early stages of embryogenesis.

with 25 and 50 nM ionomycin, but the difference was not significant between the groups (Fig 1).

Impact of ionomycin on the expression levels of genes related to cell development and differentiation (IGF1)

Gene expression of the IGF1 gene was estimated in embryos from the early stages of embryogenesis. IGF1 expression was significantly lower (P<0.05) in the groups supplemented with 50 nM ionomycin compared to that in the control and ionomycin 25 nM groups (Fig 1).

Gene expression in the late stages of embryogenesis

Impact of ionomycin on the expression levels of genes related to apoptosis (Bax and Bcl-2)

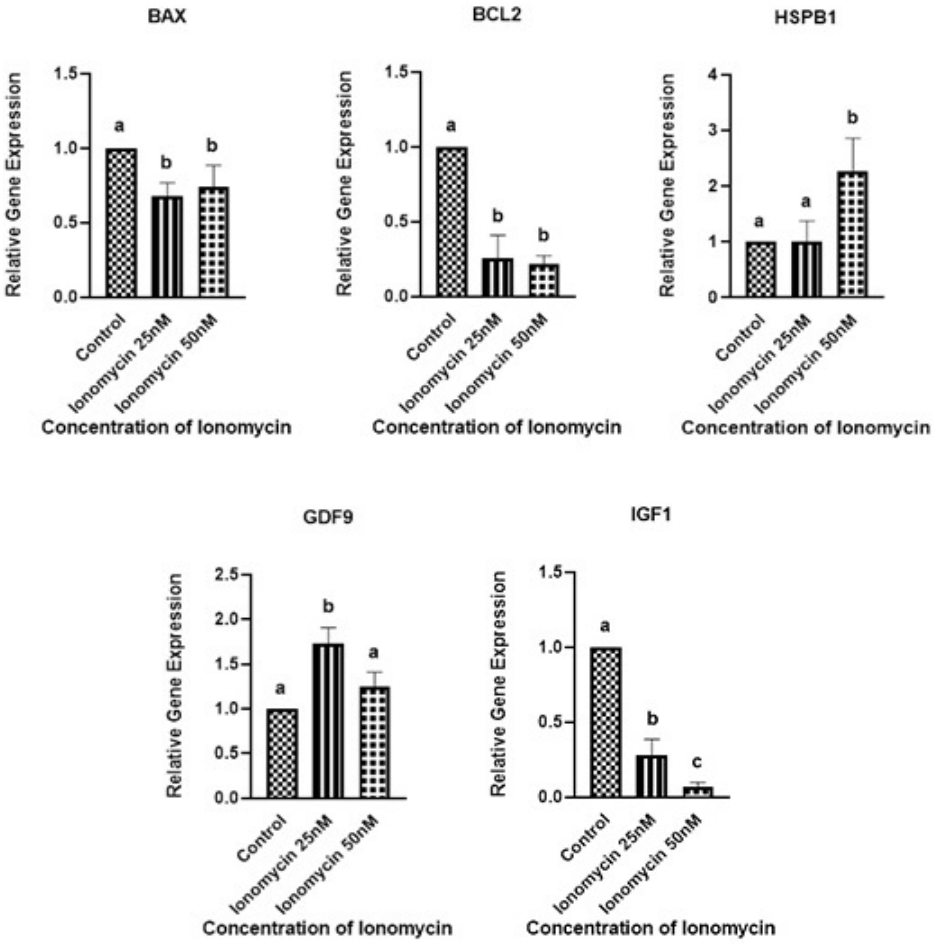
Gene expression of the BAX gene was estimated in embryos from the late stages of embryogenesis. We observed lower gene expression of the BAX gene in groups supplemented with 25 and 50 nM ionomycin. In addition, the ratio of Bcl-2 was lower in the groups supplemented with 25 and 50 nM ionomycin (P<0.05) than that in the control group (Fig 2).

Impact of ionomycin on the expression levels of genes related to stress (HSPB1)

The gene expression of HSBP1 was determined in embryos from the late stages of embryogenesis. HSPB1 expression was significantly higher (P<0.05) in the group supplemented with 50 nM ionomycin, with a non-significant difference observed between the group supplemented with 25 nM ionomycin and the control group (Fig 2).

Impact of ionomycin on the expression levels of genes related to granulosa cell development, nuclear maturation and embryo development (GDF9)

The gene expression of GDF9 was determined in embryos from the late stages of embryogenesis. GDF9 expression was a significantly higher (P<0.05) in the group supplemented with 25 nM ionomycin, with a non-significant



Data are expressed as Mean ± SEM. Different superscript (a, b, c) are significantly different within the columns, (P<0.05).

Fig 2: Impact of ionomycin on BAX, BCL-2, HSBP1, GDF9 and IGF1 gene expression ratio in bovine embryos from the late stages of embryogenesis.

difference observed between the group supplemented with 50 nM ionomycin and the control group (Fig 2).

Impact of ionomycin on the expression levels of genes related to cell development and differentiation (IGF1)

The gene expression of IGF1 was determined in embryos from the late stages of embryogenesis. The group supplied 50nM ionomycin showed significantly decreased IGF1 expression ($P<0.05$), in comparison to the control and 25 nM ionomycin groups (Fig 2).

The aim of this study was to evaluate the expression of genes (Bcl-2, Bax, HSPB1, IGF1 and GDF9) in different stages of bovine embryos produced *in vitro* by investigating the effects of applying 25 and 50 nM ionomycin to the sperm capacitation medium. This analysis was performed to elucidate the effects of treating the sperm with ionomycin before fertilization by either down regulating or upregulating of the paternal gene expiration epically in the IVEP.

For a normal developing embryo, the expression of both maternal and paternal genes is required. An intense epigenetic change occurs upon fertilization to establish pluripotency (Mclay *et al.,* 2003).

Several factors are involved in the regulation of parental genes in preimplantation embryos (Tulay, 2017).

Maternal gene expression was activated in the early embryo cleavage stages (from the zygote until the 4 to 8 cell stage), while paternal gene expression began after the 4-8 cell stage. Such paternal effects may be mediated by paternally induced epigenetic modifications during early embryogenesis, as outline by Valleh *et al.* (2015). The genes selected for this study (BAX, BCL-2, HSP-70, GDF-9 and IGF1) are sensitive in the IVEP and might also correspond to the aims of this study. The results showed that the treated (25 and 50 nM ionomycin) sperm groups experienced variations in the expression of the apoptotic and cell death genes BAX and the Bcl-2 between the treated and the control groups during early cleavage. BAX did not show any differences. However, during late cleavage development, the control group presented higher gene expression than that in the two treatment groups. This difference possibly emerged because the gene expression of treated BAX was down regulated in the early cleavage stage and upregulated in the late cleavage stage. The Bcl-2 gene of the control group showed higher gene expression in both the early (from zygote to the 32-cell stage) and late cleavage stages (morula to blastula cleavage stages), compared to that in the treated group. The differences in the expression of this gene disagree with the results of Valleh *et al.* (2015) because this previous study used different male sperm from different breeds without any treatment of the sperm prior to fertilization. The effects of altered gene expression patterns on *in vitro* produced bovine embryos, particularly when cultured under suboptimal conditions, were reflected by the occurrence of clinically important phenomena such as apoptosis, as reported in Badr *et al.* (2007). This previous review focused on the morphogenetic embryo development and gene expression profile in IVP embryos, with emphasis on the different parameters that may alter gene expression patterns during the critical period of *in vitro* embryo culture. However, our study provides results even during the sperm capacitation time. In addition, the Bcl-2 in the more strongly treated sperm group (50 nM) was expressed more highly than that in the 25 nM ionomycin and control groups. This result agrees with other studies that showed variation in the gene expression of *in vitro* bovines during early embryo development (Yang and Rajamahendran, 2002; Kim *et al.,* 2004; Ebrahimi *et al.,* 2010; Barakat *et al.,* 2015; Al-Malahi *et al.,* 2022). In early embryo development, the less-strongly treated sperm group (25 nM ionomycin) presented lower HSP-1 gene expression than that in the control group, while the more strongly treated sperm group (50 nM ionomycin) did not show any significant differences. These results agree with those of Oliveira *et al.* (2005) who showed that Hsp70 gene expression varies between distinct stages of embryo development and embryo density levels. These differences might negatively affect the patterns of gene expression for *in vitro* produced bovine embryos. This phenomenon might have negatively affected the patterns of gene expression among *in vitro* produced bovine embryos (de Oliveira *et al.,* 2005), as well as the pH-induced changes in the structure of the heat shock protein HspB1, as indicated by Chernik *et al.* (2004).

The result of GDF-9 gene expression did not show differences between the treated and control groups in the early cleavage stage, while during the late cleavage stages, we observed higher gene expression levels in the 25nM ionomycin-treated group compared to the control and the 50 nM ionomycin-treated group. These results disagree with the findings of Tali *et al.* (2019), where GDF9 showed the highest expression in immature ovine oocytes, which decreased during maturation and embryo production. This difference might be attributable to the differences between ovine and bovine IVEP.

The present study showed higher levels of IGF1 gene expression in the control group compared to that in both treated groups; these levels decreased with an increase in the treatment dose. This result agrees with the *in vitro* production of bovine embryos, which were found to negatively affect the amount of IGF1 gene expression on day 7 (Bertolini, 2002). This result conflicts with the results for mouse IVEP, in which a significant association was found between IGF-1 expression and blastocyst formation *in vitro* ($P<0.01$) (Kowalik *et al.,* 1999). This result may be due to the epigenetic effects of the paternal gene caused by sperm treatment before fertilization and the downregulation of gene expression in bovine IGF1.

CONCLUSION

The best-quality embryos and gene expression can be determined by studying gene expression. Although an embryo's genetic material comes from equal numbers of

maternal and paternal chromosomes and genes, the transition from the maternal and paternal genome to the embryonic genome and gene expression during embryo development are controlled by several factors, including genetic makeup, epigenetic conditions, embryo feeding and culture materials. The balance between these factors can affect the development and viability of good-quality embryos. Gene expression was able to predict good-quality embryo development, but other advanced techniques, such microarrays, could also be used to provide functional markers for the best-quality IVEP.

ACKNOWLEDGEMENT

The authors sincerely acknowledge the Researcher Support Project (RSP-2024/R232) for funding this work at King Saud University, Riyadh, Saudi Arabia.

Conflict of interest

All authors declared that there is no conflict of interest.

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