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The Role of Ionomycin in the Improvement of *in vitro* Fertilization and Increasing the Rate of Bovine Embryo Development

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ABSTRACT

Background: The utilization of *in vitro* fertilization (IVF) and *in vitro* embryo production (IVEP) has increased significantly as an effective tool of assisted reproductive technology. Sperm capacitation is a process of membrane maturation allowing the acrosome reaction to take place. Ionomycin is a calcium ionophore that induces the acrosome reaction by increasing calcium cycling across phospholipid membranes. The aim of this study was to enhance the cleavage rate and development of bovine embryos.

Methods: Bovine ovaries were collected from the central slaughter house. Oocytes were obtained from follicles and were let to mature for 24 h. Frozen bull semen thawed in 37°C water and transferred to a discontinuous percoll gradient (Sigma GE17-0891-01). The spermatozoon pellet was separated into four groups after resuspension in bovine *in vitro* fertilization media (BO-IVF): control without additives; with DMSO and with ionomycin (25 and 50 nM) and kept in an incubator for three hours. The oocytes matured under commonly used media (TCM-199) for 24 h. The mature oocytes were then fertilized for 18-24 h. The embryos were cultured in SOF medium until the seventh day to study cleavage rate and blastocyst rate.

Result: The results indicated that 50 nM ionomycin supplementation considerably reduced cleavage while DMSO and 25 nM ionomycin supplementation had no effect on cleavage percentage. Moreover, when compared to the other groups, ionomycin at 25 nM significantly increased blastocyst development. In conclusion, the highest rate of blastocyst development was observed in the capacitation medium supplemented with 25 nM ionomycin, apparently due to the importance of calcium and metabolism in spermatozoa for fertilization and embryo development.

Key words: Bovine *in vitro* maturation, Embryo production, *In vitro* fertilization, Ionomycin.

INTRODUCTION

In vitro embryo production is a crucial technique in cow breeding that has seen a substantial increase in utilization. Commercial companies produce bovine embryos throughout the world (Abd El-Aziz *et al*., 2016; Stoecklein *et al*., 2021; Blaschka *et al*., 2021).

A certain protocol is taken during the *in vitro* production of embryos to mimic the *in vivo* conditions in various types of animals, beginning with *in vitro* maturation of oocytes within 20 to 24 hours following retrieval (Wrenzycki, 2018; Damayanti *et al*., 2020) and the first polar body extrusion, which is required for fertilization and the beginning of embryonic development (Sirard, 2016; Turhan *et al*., 2021). Therefore, the oocyte's maturation is essential for fertilization and preimplantation development (Barakat *et al*., 2018).

The second stage of *in vitro* production, known as *in vitro* fertilization, is characterized by the creation of male and female pronuclei and the extrusion of the second polar body (Parrish, 2014). For the *in vitro* fertilization process to begin, capacitated acrosome reaction of spermatozoa must be found beside mature oocytes, such sperm has the capacity to break through the zona pellucida of the oocyte and merge with the ooplasm during the fertilization process (Stival *et al*., 2016).

Capacitation is believed to be primarily brought on by changes in cholesterol concentration, lipid composition, calcium permeability, fluidity and surface characteristics of membranes (Davis, 1981). Most of these alterations are connected to modifications in spermatozoa's plasma membrane, supporting the theory that capacitation is a process of membrane maturation (Sukardi *et al*., 2001).

The *in vitro* capacitation of the spermatozoa is achieved by washing the sperm in a media containing equal levels of electrolytes, metabolic energy sources and compounds that affect the sperm's vitality and motility as well as the induction and pace of the acrosome reaction process. Some of the substances that contribute to the motility and vitality of sperm include β-amino acids (*e.g*. hypotaurine and taurine), catecholamines (*e.g*. adrenaline), as well heparin and

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pentoxifylline, while caffeine stimulates hyperactivation and the acrosome reaction (Leibfried and Bavister, 1982; Parrish *et al*., 1988; Taketo *et al*., 1991; Pereira *et al*., 2000; Barakat *et al*., 2015). The calcium ionophore has been the most widely used agent for triggering the acrosome reaction *in vitro*. Triana *et al*. (1980) were the first to discover that calcium ionophore A23187 causes changes in bovine sperm depending on the calcium ion.

Ionomycin is a calcium ionophore that increases Ca^{2+} cycling across phospholipid membranes to induce the acrosome reaction (Ball *et al*., 1983). With the help of the calcium ionophore A23187, spermatozoa from a number of species, including horses, can be made to go through the acrosome reaction (Varner *et al*., 1987; Zhang *et al*., 1991; Magistrini and Palmer, 1990; Farlin *et al*., 1992).

The *in vitro* culture of bovine embryos, which initiates with zygote culture and typically takes around 7 to 8 days, is the last stage of *in vitro* production (IVP). The embryo advances through its first cleavage division, the embryonic genome is activated and the morula and blastocyst are formed at this stage (Wrenzycki, 2018; Ramos-Deus *et al*., 2020; Nogueira *et al*., 2021). This study aims to improve the cleavage rate and development of bovine embryos in order to increase the efficiency of *in vitro* fertilization by investigating the effects of applying DMSO and (25, 50 nM) ionomycin to the sperm capacitation medium.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and media used in this study were from Sigma Corporation (St. Louis, MO, USA) unless otherwise noted.

Experimental design

All of the experiments were conducted in the Zoology Department of the College of Science at King Saud University from January to June. We had four different groups to be examined; each group had seven replicates. All groups had the same final volume and final sperm cell concentration (8×10^6 sperm per ml) and the only difference between them was the final composition of the capacitation medium. The standard capacitation medium consisted of a Bracket-Oliphant medium (112 mM NaCl, 4 mM KCl, 2.25 mM $CaCl_2.2H_2O$, 0.5 mM $MgCl_2.6H_2O$, 37 mM $NaHCO_3$ and 0.83 mM $NaH_2PO_4.2H_2O$, 1.25 mM Sodium Pyruvate and 0.05 mM Phenol Red) supplemented with 10 µg/ml Heparin sodium salt, 3 mg/ml BSA and 0.5 µg/ml gentamicin sulfate. The first group was the control group; the sperm were capacitated using a standard capacitation medium without any additives. The second group was the DMSO group; the sperm were capacitated using a standard capacitation medium supplemented with DMSO solution. The third group was the low-treatment group; the sperm were capacitated using a standard capacitation medium supplemented with ionomycin at 25 nM final concentration. The sperm were capacitated using a standard capacitation medium supplemented with ionomycin at a final concentration of 50 nM in the last group, development assessment was used at the end of seven days of culturing.

Ionomycin solution

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

Frozen semen

Frozen semen of Holstein Friesian cattle was obtained from the Center of Asset Conservation and Genetic Improvement in Al-Kharj City, which is affiliated with the Saudi Ministry of Environment, Water and Agriculture.

Oocyte collection and *in vitro* maturation (IVM)

Ovaries were collected from the central slaughterhouse in Riyadh (Saudi Arabia) and transported to the laboratory in 0.9% sodium chloride (NaCl) within 2 hours. The follicles were separated into three groups according to their diameter: small (S: 3 mm), medium (M: 3-6 mm) and large (L: >6 - <10 mm). Through the use of the aspiration method, the oocytes were extracted from each of the three types of follicles. using a sterile syringe on 0.5 ml of TCM199 handling medium (Hank's salts) supplemented with 50 µg/ml gentamicin sulfate, 0.5 mM sodium pyruvate and 10% Fetal Bovine Serum (FBS). Oocytes with three or more layers of intact cumulus cells and uniform cytoplasm were collected using a mouth pipette under a stereomicroscope. The cumulus-oocyte complexes were then washed three times in 100 µl of TCM-199 maturation medium (Earle's salts) (Sigma M4530; Merck KGaA, Darmstadt, Germany) with 0.5 mM Na-Pyruvate, 0.023 IU/ml luteinizing hormone (LH) (Sigma L5269), 0.02 IU/ml follicle-stimulating hormone (FSH) (Sigma F8174), 1 µg/ml Estradiol (E2) (Sigma E2758), 50 µg/ml gentamicin sulfate and 10% FBS. Then, groups of ten cumulus-oocyte complexes were transferred to 60 mm Petri dishes in 50 µl maturation drops which were covered with mineral oil and incubated for 24 hours in a humidified atmosphere of 5% CO_2 at 39°C. (Ammari *et al*., 2022a).

In vitro fertilization (IVF) of oocytes

The sperm capacitation medium used in this trial consisted of a Bracket-Oliphant medium (112 mM sodium chloride, 4 mM potassium chloride, 2.25 mM calcium chloride dihydrate, 0.5 mM magnesium chloride hexahydrate, 37 mM sodium bicarbonate, 0.83 mM sodium dihydrogen phosphate dehydrate and 0.05 mM phenol red) supplemented with 10 µg/ml heparin sodium salt, 3 mg/ml BSA, 1.25 mM sodium pyruvate and 0.5 µg/ml gentamicin sulfate and was subsequently incubated at least two hours before being used.

Frozen bull semen thawed in 37°C water and transferred to a discontinuous percoll gradient (Sigma GE17-0891-01) [45 and 90% (vol/vol) Percoll] (Maziero *et al*., 2020; Nogueira *et al*., 2021). The semen was placed in a 15 mL tube containing 5 ml of capacitation medium. The pooled semen was washed twice to get rid of ionomycin residues present in the capacitation medium before fertilization with

capacitation medium by centrifugation at 1800 rpm for 5 min. The resultant pellet was diluted to give a concentration of 8×10^6 sperm per ml and then split into four different experimental treatments: The first treatment was the control, the sperm were capacitated using a capacitation medium without any additives; the second was the DMSO group, the sperm were capacitated using a capacitation medium supplemented with DMSO solution; the third was the low-treated group, the sperm were capacitated using capacitation medium supplemented with ionomycin as 25 nM final ionomycin concentration; the last group was the high-treated group, the sperm were capacitated using capacitation medium supplemented with ionomycin as 50 nM final ionomycin concentration. Before transfer to fertilization drops, the oocytes were washed four times in the previous capacitation medium (BO-IVF). Insemination was carried out by adding 8×10^6 into the fertilization medium that included 10 oocytes per 50 µl fertilization drop in an atmosphere of 5% CO_2 and humidified air at 39°C for 24 h.

In vitro culture (IVC) of embryos

A glass Pasteur pipette (Thermo Fisher,15202699) was used to repeatedly pipette zygotes in a hyaluronidase enzyme solution at 24 hours after fertilization to completely eliminate cumulus cells. The zygotes were subsequently washed in an *in vitro* culture medium (IVC-SOF) (Caisson IVL05) enriched with 1 mM L-glutamine, 0.34 mM sodium pyruvate, 1% (v/v) 50X MEM-essential amino acids (Sigma B6766), 1% (v/v) 100X MEM nonessential amino acids (Sigma M7145), 25 µg/ml gentamicin, 3 mg/mL BSA (Sigma A6003), 1 µg/ml EDTA and 1.5 mM glucose. They were then assigned to 50 µl drop of SOF medium (25 embryos/drop) in a 35 mm Petri dish (BD Falcon, 351008) and the drops were covered with mineral oil (Sigma M 5310). Embryos were maintained in incubation for 7-8 days at 39°C in a high-humidity environment with 5% CO_2, 5% O_2 and 90% N_2 (Ammari *et al*., 2022b). The cleavage rate and blastocyst rate were calculated using the following equations at the end of the seventh day of embryo culture:

$$\text{Cleavage rate} = \frac{\text{No. of cleaved oocytes}}{\text{No. of cultured oocytes}}$$

$$\text{Blastocyst rate} = \frac{\text{No. of blastocysts}}{\text{No. of cleaved embryos}} \times 100$$

Statistical analysis

The Mine Tab INSTAT program was used to analyze all results while Chi-square test was carried out to analyze the cleavage and blastocyst rates (Ammari *et al*., 2022b).

RESULTS AND DISCUSSION

Embryo cleavage rate

The impact of incorporating DMSO and ionomycin into the spermatozoa's *in vitro* fertilization medium on the embryo cleavage rate following oocyte fertilization was the focus of the current investigation.

The embryo cleavage rate was significantly (P<0.05) higher in control (67.1%), DMSO (66.3%) and ionomycin 25 nM (69.5%) groups compared to ionomycin 50 nM (56.8%), as shown in Table 2.

The average number of embryos during the stage of development

The number of embryos at each cleavage stage was determined at the end of the seventh day of embryo culture. Degenerated oocytes in the 50 nM ionomycin group (17.8%), were significantly higher (P<0.05) than in control (13.3%), DMSO (13.5%) and ionomycin 25 nM (13.6%) groups. Additionally, the experimental 25 nM ionomycin group had a significantly (P<0.05) lower percentage of non-cleavage oocytes (one cell) than the 50 nM ionomycin group (16.9% vs. 25.4%). Furthermore, the percentage of fragmented embryos in both the control group (18.2%) and the DMSO group (18.1%) was significantly higher (P<0.05), than both 25 nM ionomycin (9.8%) and 50 nM ionomycin (11.7%) groups. Most of the embryos in the 50 nM ionomycin group (14.0%) were in the 2-cell stage but the blastocysts were on the same level, which was significantly higher (P<0.05) compared to the control (3.7%), DMSO (4.0%) and 25 nM ionomycin (2.1%) groups. As for the 4-cell stage, it was significantly (P<0.05) lower in the experimental 25 nM ionomycin group (2.1%) compared to the experimental 50 nM ionomycin group (6.0%). In addition, the blastocyst was significantly (P<0.05) higher in the 25 nM ionomycin group (71.5%), compared to the control (50.6%), DMSO (51.4%) and 50 nM ionomycin (36.9%) groups. As demonstrated in Table 1, there was no statistically significant difference between any of the groups regarding the percentage of embryos at the 8 and 16-cell stages, as well as at the morula stage.

Embryo arrival rate to the blastocyst

The current study examined the impact of the addition of DMSO and ionomycin to IVF medium on the cleavage rate and blastocyst rate on the seventh day from embryo culture.

In this experiment, the blastocyst rate was significantly (P<0.05) higher in the 25 nM ionomycin group (71.5%), compared to the control (50.6%), DMSO (51.4%) and 50 nM ionomycin (36.9%) groups. In addition, the expanded blastocyst rate was significantly (P<0.05) higher in the 25 nM ionomycin group (34.5%), compared to control (22.3%), DMSO (22.7%) and 50 nM ionomycin (14.0%) groups. In addition, the rate of hatching blastocyst was significantly (P<0.05) higher in the 25 nM ionomycin group (20.9%), compared to control (13.3%), DMSO (13.0%) and 50 nM ionomycin (11.2%) groups. In addition, as shown in Table 2 and Fig 1, the control (15.0%), DMSO (15.7%) and 25 nM ionomycin (16.2%) groups all had significantly (P<0.05) greater rates of hatched blastocysts than the 50 nM ionomycin group (11.7%).

The capacitation process requires the removal of inhibitors from the sperm head, an increase in intracellular pH and a decrease in the cholesterol/phospholipid ratio of the membranes, allowing them to be more permeable to calcium ions (Langlais and Roberts, 1985). Our study

objective is to improve the efficiency of *in vitro* fertilization by increasing the cleavage rate and development of bovine embryos by studying the effect of adding; DMSO, 25 nM and 50 nM ionomycin to the sperm capacitation medium. The results of this study contradict those of Hong *et al*. (2020), who utilized 5 mM ionomycin to the *in vitro* maturation medium and goat ova embryo development and discovered no significant differences between the experimental group and the control group. This might be a result of the differing ionomycin concentrations they employed in their investigation or because they added the ionomycin to the maturation medium rather than the capacitation medium.

Table 1: Effect of DMSO and ionomycin addition to the IVF medium on embryo development of bovine embryos (seven replicates).

Treatment	No. of cultured oocytes	Degenerated oocytesn (%)	One celln (%)	Fragmented embryosn (%)	2 celln (%)	4 celln (%)	8 celln (%)	16 celln (%)	Morulan (%)	Blastocystn (%)
Control	347	46 (13.3)^{b}	68 (19.6)^{ab}	63 (18.2)^{a}	13 (3.7)^{b}	13 (3.7)^{ab}	14 (4.0)	8 (2.3)	4 (1.2)	118 (50.6)^{b}
DMSO	326	44 (13.5)^{b}	66 (20.2)^{ab}	59 (18.1)^{a}	13 (4.0)^{b}	13 (4.0)^{ab}	12 (3.7)	6 (1.8)	2 (0.6)	111 (51.4)^{b}
Ionomycin (25 nM)	338	46 (13.6)^{b}	57 (16.9)^{b}	33 (9.8)^{b}	7 (2.1)^{b}	7 (2.1)^{b}	10 (3.0)	7 (2.1)	3 (0.9)	168 (71.5)^{a}
Ionomycin (50 nM)	315	56 (17.8)^{a}	80 (25.4)^{a}	37 (11.7)^{ab}	44 (14.0)^{a}	19 (6.0)^{a}	8 (2.5)	3 (1.0)	2 (0.6)	66 (36.9)^{c}

^{a,b} Different superscripts within columns are significantly different (P<0.05).

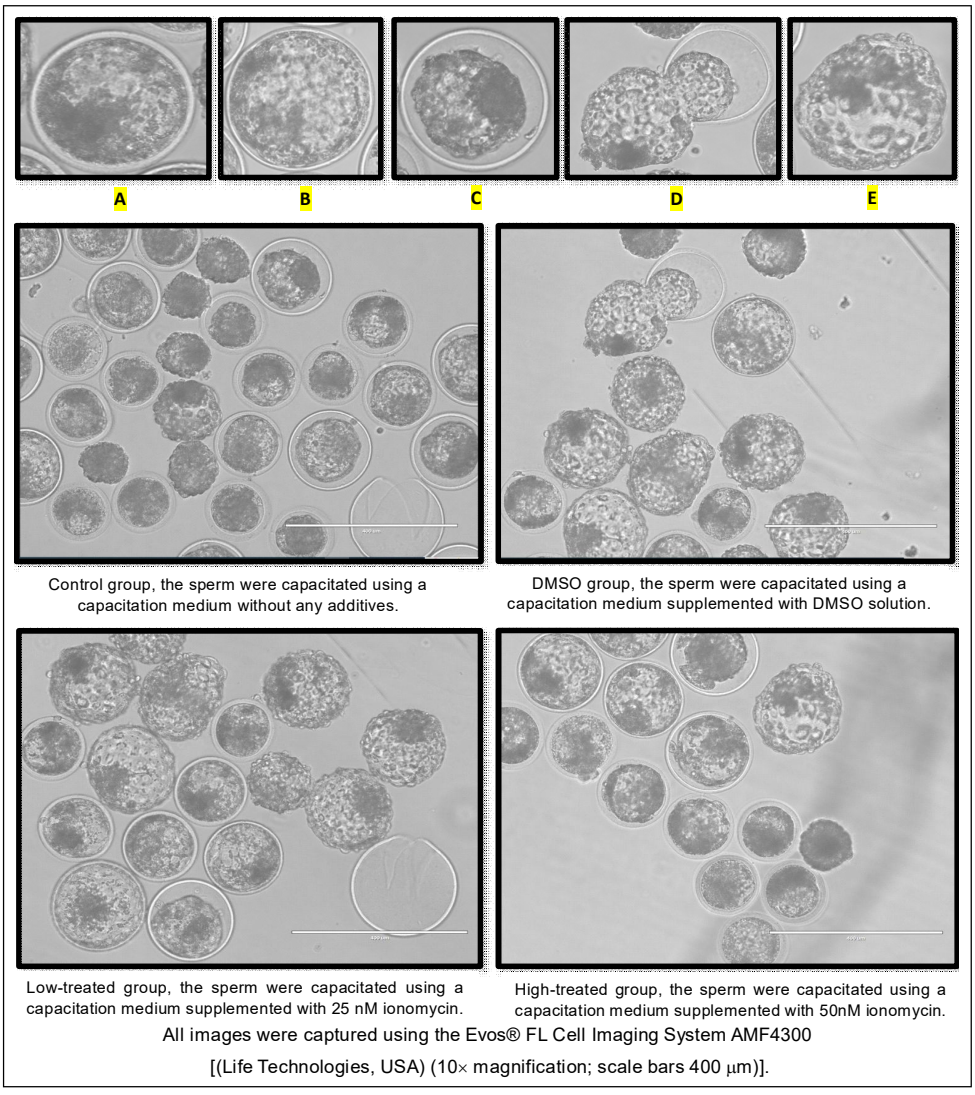

Fig 1: The different stages of embryo blastocyst development in the experiment.

A: Early blastocyst, B: Expanded blastocyst, C: Before hatching blastocyst, D: Hatching blastocyst, E: Hatched blastocyst.

Table 2: Impact of DMSO and ionomycin addition to the IVF medium on cleavage rate and blastocyst rate of bovine oocytes (seven replicates).

Treatment	No. of cultured oocytes	No. of cleaved oocytes (cleavage rate) n (%)	No. of expanded blastocystn (%)	No. of hatching blastocystn (%)	No. of hatched blastocystn (%)	No. of blastocysts (Blastocyst rate) n (%)
Control	347	233 (67.1)^{a}	52 (22.3)^{b}	31 (13.3)^{b}	35 (15.0)^{a}	118 (50.6)^{b}
DMSO	326	216 (66.3)^{a}	49 (22.7)^{bc}	28 (13.0)^{b}	34 (15.7)^{a}	111 (51.4)^{b}
Ionomycin (25 nM)	338	235 (69.5)^{a}	81 (34.5)^{a}	49 (20.9)^{a}	38 (16.2)^{a}	168 (71.5)^{a}
Ionomycin (50 nM)	315	179 (56.8)^{b}	25 (14.0)^{c}	20 (11.2)^{b}	21 (11.7)^{b}	66 (36.9)^{c}

$^{a, b, c:}$ Different superscripts within columns are significantly different ($P<0.05$).

Although it was discovered that ionomycin activated mouse oocyte and embryo development (Heytens *et al*., 2008). Additionally, according to Navarrete (2017), the calcium and metabolism in sperm are thought to play a key role in fertilization and embryo development. While our result agrees with Slonina *et al*. (1995) who used the Ca^{2+} A23187 for stallion sperm capacitation and found that the sperm exposed to A23187 had a significantly higher occurrence of acrosome reactions than sperm capacitated without Ca^{2+} A23187 ($p<0.01$).

Increased Ca^{2+} influx brought on by the acrosome reaction (AR) leads to higher Ca^{2+} concentrations inside cells. This increase in Ca^{2+} enables dephosphorylation/ activation of p-gelsolin resulting in the dispersion of F-actin, allowing the plasma membrane PM and outer acrosome membrane OAM to come into close proximity and undergo fusion (Breitbart and Shabtay, 2018).

The sperm capacitation process requires protein kinase A activation (PKA), changes in membrane potential and an increase in intracellular calcium (Ca^{2+}). Inhibition of these pathways results in loss of fertilizing ability *in vivo* and *in vitro*. According to Nakagawa *et al*. (2001) and Lu *et al*. (2006), the greater concentration (50 nM) of ionomycin may increase the likelihood of danger in sperm *in vitro* capacitation, IVF and/or embryo development, necessitating further research. Despite the lack of conclusive proof of calcium ionophore toxicity on gametes and embryos, given the widespread application of the treatment in human oocytes, there could still be some concerns. However, according to Spandorfer *et al*. (2001), the main cause of IVF failure seems to be the sperm's incapability to penetrate the oocyte membrane, which demands for further research.

CONCLUSION

Ionomycin, at a dose of 25 nM in capacitation medium, performed better when compared to the other implemented groups, resulting in a faster rate of early embryo cleavage development. Moreover, the medium treated with 25 nM ionomycin showed the highest rate of blastocyst formation. In contrast to DMSO and control, which had no effect on the percentage of cleavage at the end of the experiment, 50 nM ionomycin supplementation significantly reduced cleavage.

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Conflict of interest: None.

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