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Effect of oocyte quality and activation protocols on bovine embryo development following intracytoplasmic sperm injection

Ömer KORKMAZ[1,*], Şükrü KÜPLÜLÜ[2], Yüksel AĞCA[3], İbrahim Mert POLAT[2]

[1]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Harran University, 63040 Eyyubiye, Şanlıurfa, Turkey
[2]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey
[3]Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri, USA

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Abstract: The purpose of this study was to investigate the effects of oocyte quality and activation protocols on the in vitro developmental competence of bovine embryos after intracytoplasmic sperm injection (ICSI). Bovine oocytes were grouped as being of excellent, good, and poor quality. All of the oocytes were activated using a calcium ionophore only, ethanol only, and 6-dimethylaminopurine (6-DMAP) following calcium ionophore. For the excellent quality oocytes, cleavage rates after ICSI were 70% in the Ca ionophore + 6-DMAP activation group, 47% in the ethanol activation group, 41.82% in the Ca ionophore group, and 30% in the nontreated control group. Cleavage rates for the good quality oocytes after activation with these different agents were 31%, 16.03%, 19.8%, and 12.9%, respectively, while the rates for the poor quality oocytes were lower (7%, 6%, 2%, and 1%, respectively). In conclusion, using Ca ionophore + 6-DMAP improves the in vitro developmental competence for bovine embryos after ICSI. Furthermore, the results for the excellent quality oocytes indicate the importance of the preselection of the oocytes and activation methods.

Key words: Bovine oocyte, calcium ionophore, ethanol, intracytoplasmic sperm injection, 6-dimethylaminopurine

1. Introduction

Intracytoplasmic sperm injection (ICSI) is a useful assisted reproductive technique by which a single spermatozoon is directly injected into the ooplasm with the help of a microinjector in the case of poor sperm motility or sperm count (1,2). The possibility of ICSI in bovine oocytes was first shown in 1984 (3). In humans, ICSI is widely used in male infertility. To understand the long-term effects of ICSI, systematical experiments in animals have been reported (4,5).

The most common problems in the ovine ICSI technique are the low oocyte activation rate after microinjection and the low live embryo rate. Therefore, the activation of bovine oocytes by different treatment methods should be investigated (6). Normally, sperm-triggered activation is the most important stage of embryonic development. The mammalian sperm–oocyte fusion increases intracellular free Ca^{2+} concentration and stops histone H1 kinase activity. This causes the production of receptor-sourced inositol-1,4,5-triphosphate (IP3) via G proteins and tyrosine kinase. The receptor in the mammalian oocyte supporting this theory is the source of IP3's reputation (7–10). With a specific receptor, IP3 stimulates IP3 receptor type 1. This causes an increase in the free Ca^{2+} released from the endoplasmic reticulum (11). The other hypothesis explains the trigger of Ca^{2+} released by the interactions of some structures in oocyte cytoplasm, and by soluble sperm factor from sperm during gamete fusion. Sperm also includes Ca^{2+}-releasing structures such as IP3 and phospholipase C or phospholipase activator (7,10).

Calcium ionophore is one of the most frequently used artificial factors for oocyte activation and for balancing the maturation supporting factor (maturating promotion factor). Ethanol has been reported to stimulate the activation of metaphase II oocytes and lead to IP3 stimulation from the plasma membrane in the later stages of embryos. Combined oocyte activation treatment is done by phosphorylation or protein synthesis inhibitors. For this purpose, the histone H1 kinase inhibitor Ca ionophore is used following 6-dimethylaminopurine (6-DMAP) in bovine oocytes. The effects of 6-DMAP on chromatin and microtubule configurations during meiosis are mediated by the inhibition of protein kinases and, consequently, protein phosphorylation (12). Many different activators have been tested; however, there are few reports that systematically compared the activation

* Correspondence: ahuramazda@harran.edu.tr

of the oocytes in one particular species. The availability of this information would be very valuable for use in basic reproductive biology, such as ICSI and nuclear transfer (7).

In this study, we intended to determine the optimal chemical activators after bovine ICSI on embryonic development potential for oocytes of various qualities.

2. Materials and methods

2.1. Oocyte recovery and classification

Ovaries were obtained from a local slaughterhouse and brought to the laboratory within 3 h in a thermos containing saline solution at 37 °C with 100 IU/mL penicillin and 100 mg/mL streptomycin. For aspiration, peripheral follicles of 2–8 mm in diameter were selected. The aspiration process was performed using 10-mL plastic syringes fitted with 18-G needles. Oocytes used in the study were classified in a manner similar to that described by Brackett and Zuelke (13).

2.2. In vitro maturation

Maturation media (TCM 199) was prepared by adding 1 mL of solution including 100 IU penicillin, 100 mg streptomycin, and 100 ng/mL epidermal growth factor (pH 7.2–7.4, 270–295 mOsm) to 3 mg/mL bovine serum albumin (BSA)-added TCM 199 solution. Every drop used in the study was covered with mineral oil. For the maturation, activation, and embryo culture, a 5% CO_2 atmosphere incubator set to 38.5 °C was used. The swim-up procedure was applied in the selection of motile spermatozoon.

2.3. Intracytoplasmic sperm injection

After 24 h of oocyte maturation, extrusion of the first polar body and total cumulus expansion rates were recorded. Sperm microinjection was carried out with the help of an inverted microscope hardware (DMIL, Leica, Germany) micromanipulator system (MMN-1, NARISHIGE, Japan), following the oocyte cumulus cell removal process. All of the oocytes were put into droplets containing 5 µL of in vitro fertilization-Tyrode's albumin lactate pyruvate (IVF-TALP). Sperm were transferred into 10 µL of sperm culture medium (Sp-TALP) containing 10 µg/mL heparin. Before the sperm injection, 4% polyvinylpyrrolidone (PVP) solution was used to slow down spermatozoon movement, which was achieved by breaking the tail to stop the rounding movement with the help of the tip of a sperm injection needle. With the help of the holding pipette, when the polar body was located in a 6 or 12 o'clock position, the zona pellucida and oolemma were simultaneously punctured in the 3 o'clock position using an injection needle containing spermatozoa. Approximately two-thirds of the oocyte was exceeded and some part of the retreated ooplasm and sperm were injected into the ooplasm.

2.4. Oocyte activation

Sperm-injected oocytes were treated with 3 different substances: 1) Ca ionophore: the oocytes were activated for 5 min with 5 µM Ca ionophore prepared in phosphate buffered saline (PBS) without Ca-Mg and then washed 3 times with 5% calf serum added to TCM 199; 2) combined activation with 6-DMAP following Ca ionophore: bovine oocytes were cultured in 5% calf serum added to TCM 199 for 3 h after being activated with 5 µM Ca ionophore added to PBS (without Ca-Mg) for 5 min, and the oocytes were then incubated in 1.9 mM 6-DMAP prepared in PBS for 3 h; 3) ethanol: the oocytes were activated in 7% ethanol prepared in TCM-199 including 1 mg/mL PVP after being cultured in 5% calf serum added to TCM-199 for 4 h.

In each control group, each quality of oocyte (excellent, good, and poor) was transferred to the embryo culture after ICSI without any further treatment.

2.5. Cleavage and blastocyst development

The injected oocytes (10 oocytes in each group) were cultured in Charles Rosenkrans 1 with amino acids (CR1aa) media supplemented with 3 mg/mL BSA for 5 days. On day 5, they were transferred to fresh CR1aa media containing 5% calf serum. The injected and activated oocytes were monitored for approximately 48, 120, and 168 h for first cleavage, morula formation, and blastocyst formation, respectively.

2.6. Statistical analyses

The effects of different qualities of bovine oocytes and different activation methods on embryonic development were evaluated by multiple logistic regression analysis and the Hosmer-Lemeshow H^+ statistics described in SPSS 14.01. The mean differences in the maturation criteria of different quality oocytes were compared using chi-square analysis and the same statistical package program.

3. Results

3.1. Maturation rates of different qualities of oocytes

In this study, 420 excellent, 400 good, and 415 poor quality oocytes were used. After the maturation process, the first polar body formation and the cumulus cell expansion criteria were noted according to the maturation rates of excellent, good, and poor quality oocytes; these are given in Table 1.

3.2. Cleavage, morula, and blastocyst rates

The first cleavage, morula development, and blastocyst formation rates of different activation methods used for different qualities of oocytes are given in detail in Table 2.

4. Discussion

In this study, the activation of excellent quality oocytes with Ca ionophore was lower in terms of cleavage rates than in studies reported by Chen and Seidel (14), Oikawa

Table 1. Cumulus expansion and first polar body excursion rates of different quality oocytes after maturation.

Oocyte quality	Oocyte number	Total cumulus expansion	First polar body excursion
Excellent	420	396 (94.25%)[a]	376 (89.52%)[a]
Good	400	251 (62.75%)[b]	223 (55.75%)[b]
Poor	415	0 (0%)	6 (1.45%)[c]

a, b, c: the differences between groups bearing different letters in the same column are significant ($P < 0.05$).

et al. (15), Keskintepe and Brackett (16), and Keskintepe et al. (17). The developmental potential in terms of morula and blastocyst formation rates was similar to or higher than that reported by Suttner et al. (18), Skrzyszowka et al. (19), Rho et al. (20), Li et al. (21), and Ock et al. (22).

The cleavage rates of the excellent quality oocytes following combined activation were lower than those of Suttner et al. (18), Li et al. (23), and Oikawa et al. (15) and were similar to those of Keskintepe et al. (17) and Ock et al. (22), but were higher than those of Rho et al. (20). While the morula formation rate was higher in the above studies, in this study, the blastocyst rate was lower than that of Oikawa et al. (15) and was parallel to those of Suttner et al. (18) and Keskintepe and Brackett (16), but was higher than those of Ock et al. (22) and Li et al. (23).

In this study, the cleavage and blastocyst formation rates were higher than those of Chen and Seidel (14) and Wei and Fukui (24) but lower than those of Oikawa et al. (15) based on the results for ethanol-activated excellent quality oocytes.

We think that the reason for the differences in our cleavage, morula, and blastocyst rates from those in the above studies could be explained by the concentration of chemicals and the timing of the oocyte activation. The results also suggest that an activation dose of Ca ionophore (5 μM vs. 10 μM) may lead to different results in terms of cleavage, morula, and blastocyst formation rates (17).

It has been reported that ICSI affects the embryonic development stages as well as the procedural differences (18). In a study conducted by Wei and Fukui (25), it was reported that without using any activators after a piezo microinjection, 171 of 256 (71.8%) oocytes cleaved and 54 of 256 (22.7%) oocytes reached the blastocyst stage. This study is also supported by the differences of embryonic development stages between conventional and piezo injection systems for bovine oocytes.

The postfertilization stage is one of the most important and critical points in producing in vitro embryos. The results are 90% higher in fertilization rates and around 30% higher in blastocyst formation rates in IVF studies

Table 2. Embryonic development stage by the administration of different activators in groups of different qualities of oocytes.

Oocyte quality	Groups (n)	Cleaved	%	Morula	%	Blastocyst	%
Excellent	CaD (110)	77	70	57	51.82	34	30.91
	E (100)	47	47	32	32	23	23
	Ca (110)	46	41.82	22	20	14	12.73
	K (100)	30	30	11	11	4	4
Good	CaD (100)	31	31	23	23	14	14
	E (106)	17	16.03	9	8.49	4	3.77
	Ca (101)	20	19.81	10	9.93	6	5.94
	K (93)	12	12.9	4	4.3	2	2.15
Poor	CaD (100)	7	7	3	3	1	1
	E (115)	6	5.21	2	1.74	0	0
	Ca (100)	2	2	1	1	0	0
	K (100)	1	1	0	0	0	0

CaD: Ca ionophore + 6-DMAP, E: ethanol activation, Ca: Ca ionophore, K: control.

(26). The most important reason for conducting in vitro studies is suggested to be the choice and constitution of the culture media in which embryonic development can take place. In this study, it was confirmed that transferring the oocytes after ICSI to 3 mg/mL BSA embryonic culture media with added CR1aa, and to the freshly prepared culture media supplemented with 5% calf serum on day 5, positively affected embryonic development.

The in vitro maturation conditions of bovine oocytes highly affect fertilization and embryonic development (1,13,27). Granulosa and theca cells help the oocytes to meet their needs during the meiotic stage and gain the ability to develop. These cells and the oocyte are in indirect relation by means of hormones and growth factors. Cumulus cells and the oocyte are in direct contact by means of the gap junctions where the intercellular connections take place (28). Additionally, cumulus cells have an organizing role in RNA synthesis (29). Cumulus cells play an important role in maturation and oocyte development due to the growth factors and hormones that they contain and their major role in RNA synthesis. In consideration of all of the reasons represented in this study, and parallel to the fact that the good quality oocyte maturation rates were lower compared to the excellent quality oocyte maturation rates, it is suggested that there is a decrease in maturation quality along with a decrease in the oocyte quality rates caused by the low rates in embryonic development after ICSI. Post-ICSI embryonic development was not observed in the poor quality oocytes. In a previous study, the cleavage ability of oocytes was classified according to cumulus morphology (30), and it was observed that 63.7% of the excellent quality oocytes and 29.5% of the good quality oocytes were cleaved. This rate decreased to 17.7% in the poor quality oocytes. This study supports the fact that oocyte quality has an effect on embryonic development.

For the combined activation protocols, using the excellent quality oocytes resulted in the best results (6,12,15,17,18,20,22,23). Moreover, in the present study, the best results were observed in the combined activation groups. Despite these results, it has been documented that 6-DMAP could skip the metaphase stage and restart the S phase of division in some nuclei; cause chromosomal anomalies such as mixoploidy, polyploidy, and abnormal zygote bodies; and prevent the secondary polar body (15). In addition, it has been pointed out that applying the combined activation method is laborious and time-consuming, and that Ca ionophore is an expensive chemical. However, ethanol activation, aside from disadvantages such as reaching lower levels of embryonic development than combined activation and being a time-consuming method, is a cheap and readily available chemical method.

In conclusion, this study showed that the best developmental competence for bovine oocytes after ICSI was obtained using a Ca ionophore + 6-DMAP combination and excellent quality oocytes, indicating the importance of the preselection of oocytes and activation methods.

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