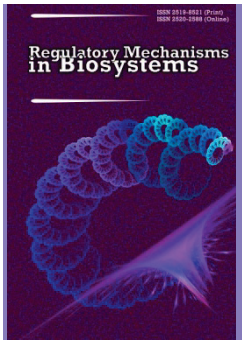

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Influence of calcium ionophore on the fertilization of bovine oocytes and their further embryonic development

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Intracytoplasmic spermatozoid injection (ICSI) is one of the commonest methods used in assisted reproductive technologies in human medicine. However, this procedure has low efficacy for bovines, mainly because of insufficient activation of oocytes after spermatozoid microinjection. One of the most effective methods of activating oocytes is considered to be the use of phosphorus calcium, though the optimal concentration of activator and its effect on pre-implant development of embyo are still open questions. An oocyte-cumulus complex of clinically healthy cows, retrieved from the ovaries during slaughter, matured over 22–24 h in *in vitro* conditions. Oocytes with visible polar body had been subjected to intracytoplasmic spermatozoid injection (ICSI), and were 15–30 min later activated in the environment with different concentrations of calcium ionophore for 15–20 min and then transferred for further cultivation in a culture medium with sodium pyruvate. The fertilization rate was identified on the second day at the 2–4th stages of cellular embryo, and the quality of obtained embyos was evaluated on day 8. Based on the statistical analysis of the data, we determined that the artificial activation of bovine oocytes using calcium ionphore after intracytoplasmic spermatozoid injection (ICSI) led to statistically significant improvement in conception and ratio of blastocytes obtained to oocytes injected. In the study, we confirmed that addition of 5, 10 and 50 μM of the agent had the same efficacy on the activation of occytes of bovine cattle. However, it has to be noted that during further cultivation of the obtained zygotes up to the blastocyte stage (day 8), we saw no significant differences in quality of embryos obtained. Therefore, use of calcium ionophore for the activation of bovine oocytes after intracytoplasmic spermatozoid injection is effective, for it promotes increase in fertilization parameters and ratio of blastocytes obtained to oocytes injected, facilitating production of higher numbers of embyos suitable for transplantation or cooling. Our previous conclusions are valuable for increasing the efficacy of methods of intracytoplasmic injection of bovine spermatozoid and its further use for purposes of science and production.

Keywords: artificial activation of oocytes; ICSI; biotechnology of reproduction of ruminants; A213187.

Introduction

Agriculture is one of the leading spheres of the Ukraine's economy, accounting for around 9.3% of the country's GDP, and providing work to almost 17% of the employable population (Jagtap et al., 2022). At the same time, an important role in it is played by animal husbandry (Herrero et al., 2013). However, agriculture is currently suffering great losses because of the Russian invasion. According to the data of the State Statistics Service of Ukraine, by January, 2022, farms of all forms of property supported a consisting of over 2.7 M head of cattle (Hladiy & Prosovych, 2022). As of now, it is impossible to identify the accurate statistical data on stock, as well as milk production. Nonetheless, according to the earlier estimates, the number of cattle of the industrial sector in the temporary occupied territories has decreased by over 15 thou individuals. The available data indicate further dynamics of decrease in the overall quantity of cows because of the problems with food supply and other components of the production sphere. The total amount of the cattle stock on fams is predicted to decrease from 423.7 to 394.2 thou individuals by early 2023 (Elfeel et al., 2022).

The agrarian sector of Ukraine mostly produces meat and dairy production for the domestic market. The country produces 99% of the poultry, pork and beef consumed by Ukrainians. Almost the entire stock of chicken, swine and cattle in Ukraine is likely to be slaughtered in the next two years. Most of those animals are commercial breeds that are not functionally different from those raised in Poland or Hungary, and therefore can be replaced quickly. However, there are traditional Ukrainian breeds such as Grey Ukrainian, Brown Carpathian, Lebedyn cattle that are already threatened. There are only few individuals abroad, and reproduction without biotechnological methods is almost impossible (Suprun & Dovha, 2021; Kovpak et al., 2022). In view of all this, postwar recovery of the livestock breeding in Ukraine is impossible without global aid, which implies adaptation and improvement of novel technologies in the sphere, and obviously financing.

Biotechnological reproduction methods with subsequent transplantation of embryos enhance the rates of reproduction of high-value animals and improve the characteristics of an existing herd (Dochi, 2019; Kovpak et al., 2022). There are two methods of obtaining embryos: *in vivo* and *in vitro*. Until 2016, *in vivo* production of cow embryos was more popular (Viana, 2020). However, as the *in vitro* sphere was developing, these methods helped obtain a higher number of embryos per animal (Hasler et al., 2003; Ferré et al., 2020). In turn, *in vitro* fertilization is carried out in two variants: coculturing of gametes (IVF) and intracytoplasmic spermatozoid injection (ICSI) (Haddad et al., 2021, Travnickova et al., 2021, Tian et al., 2022).

Intracytoplasmic spermatozoid injection (ICSI) is an *in vitro* fertilization technique when a spermatozoid is microinjected into cytoplasm of a

mature oocyte (Unnikrishnan et al., 2021). This technique was first performed in 1976 using spermatozoids and oocytes of hamsters (Uehara & Yanagimachi, 1976). Later on, the new technology has been used for other species: humans (Lanzendorf et al., 1988), cow (Goto et al., 1990), rabbits (Hosoi & Iritani, 1993), mice (Kimura & Yanagimachi, 1995), sheep (Catt & Rhodes, 1995), goats (Keskintepe et al., 1997), horses (Cochran et al., 1998) and others. However, the broad use of this method has been limited to laboratory mice (López-Saucedo et al., 2012), and the clinical use – to horses (Morris, 2018) and humans (Haddad et al., 2021).

In cattle breeding, coculturing of gametes is a more common method, because the excellent quality of gametes means higher percentage of embyos suitable for transplantation, as compared with the ICSI (Arias et al., 2015; Briski & Salamone, 2022; Fuentes et al., 2022). At the same time, ICSI is a reliable and effective reproductive technique, which demonstrates better fertilization using sex-sorted semen, with samples that have low mobility, from individuals with high genetic value, special traits (transgenic animals or threatened species) or obtained postmortem (Unnikrishnan et al., 2021). As with oocytes, ICSI can be useful for fertilizing gametes with changes that impede the fertilization (cryoconcerved oocytes, in vitro matured and retrieved from prepuberant females) (López-Saucedo et al., 2012). There are studies suggesting that the ICSI methods with the use of low-quality oocytes improve *in vitro* production of embyos, compared with the method of cocultivation of gametes (Ohlweiler et al., 2013). In addition, Ashibe et al. (2019) report that decrease in oxidative stress increases the percentage of blastocyte formaton after ICSI to 30.4% ($P < 0.05$, compared with IVF). Considering the specifics and advantages of ICSI, this method should be optimized on the territory of Ukraine.

Most researchers agree that in order to enhance ICSI efficacy for cattle, additional activation of oocytes is needed (Suttner et al., 2000, Unnikrishnan et al., 2021), because the normal fluctuations of calcium – associated with meiosis during fertilization – do not occur in this species of animals when ICSI method is used, which impedes the following development of embryo (Valencia et al., 2021). There are known different procedures of artificial oocyte activation, which are usually divided into three subtypes – mechanical, electrical and chemical stimuli (Nasr-Esfahan et al., 2010). During the mechanical activation, the plasmatic membrane of the oocyte is pierced by a microneedle in order to support calcium ingress, and after some time, ICSI is performed (Kawamura, 1939). Another method is direct microinjection of calcium into the oocyte to increase the concentration of intracellular calcium (Dirican et al., 2008). And yet despite the fact that this method effectively induces the oocyte activation, it has not become broadly used due to the complexity of execution. Electostimulation of oocytes induces the ingess of calcium through formation of pores in the plasmatic membrane. This method is efficient for the activation of bovine oocytes (Hwang et al., 2000; Hosseini et al., 2008). Periodically, repeated electrostimulation imitates the fluctuation of Ca^{2+}, which is observed during fertilization (Ozil, 1990). Also, there are data that ICSI with the piezosystem is more effective than the conventionnal method (Katayose et al., 1999), resulting in vital progeny of bovines (Horiuch et al., 2002). However, the most promising method of artificial activation of oocytes is chemical stimulation. For this purpose, there are used ethanol (Fujinami et al., 2004; Báez et al., 2021), calcium ionophore A213187 (Hosseini et al., 2008; Xu et al., 2021), ionomycin (Méo et al., 2008; Jia et al., 2023), puromycin (De Sutter et al., 1992; de Castro et al., 2022), strontium chloride (Kim et al., 2014; Fawzy et al., 2018), phorbol ester (Cuthbertson & Cobbold, 1985; Zhao et al., 2014), thimerosal (Kumbha et al., 2020), dehydroleukodine, ionomycin, anisomycin, cycloheximide, 6-dimethylaminopurine (Vichera et al., 2002), alone and in combinations. Addition of those compounds is mostly oriented at mobilization of intracellular calcium by depletion of its reserves, release of intracellular reserves of calcium and promotion of ingress of extracellular calcium ions inside the cell. Some of those compounds (ethanol, ionophore calcium A213187, ionomycin) induced increase in calcium level in oocytes, and the other compounds (strontium chloride, phorbol ester, and thimerosal) caused even greater increase (Alberio et al., 2001). However, the use of chemical compounds for artificial activation of oocytes is still limited to the model animals and reports about separate cases. Nevertheless, all of them are oriented at imitation of the natural scheme of increase in calcium after penetration by the spermatozoid and optimization of oocyte activation rates.

Empirical use of the methods of artificial activation of oocytes has demonstrated initial success in solving the problem of assisted reproductive technologies. These methods are used in attempts to increase calcium level after fertilization, but their effectiveness and safety are still in the early stages of research. As we see, there is currently a large amount of data on the *in vitro* methods of activation of oocytes of cattle, though they are significantly fragmented, and the conclusions drawn by researchers are contradicting. Nonetheless, most experiments revealed that artificial activation of bovine oocytes by calcium ionophore increased intracellular free calcium, imitatiting the physiological mechanisms that activate oocytes (Suttner et al., 2000; Wang et al., 2008; Acar & Bastan, 2011). However, there are still no generally accepted methods and concentrations of the indicated compound available for application. Therefore, this study was performed so as to analyze the influence of artificial activation of oocytes by calcium ionophore (A23187) using various concentrations of it on fertilization and further development of bovine embryos after intracytoplasmic injection of spermatozoid in order to introduce the results into practice.

Materials and methods

Ethical principles. The studies were performed at the National University of Bioresources and Nature Use of Ukraine, at the Scientific-Research Laboratory, the Center of Reproductive Animals with of Sperm and Embryo Bank in 2022–2023. The experiments were carried out according to the requirements of the "General Ethical Principles of Experiments on Animals", approved by the National Congress of Bioethics, which adhere to the positions of the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Strasbourg, 1986).

Production and maturation of oocyte-cumulus complexes. The ovaries were gathered at a slaughter house from clinically healthy cows and delivered to the laboratory. The transportation to the laboratory took no more than 3 h since the selection, the material was kept at the temperature of 30–33 °C. The operations with the ovaries were carried out in a laminar box. They were rinsed 4 times with a sterile solution, heated up to 37–38 °C, consisting of the Dulbecco's phosphate-saline buffer (Sigma, USA) with addition of kanamycin sulfate antibiotic (Sigma, USA) at the dose of 0.075 mg/cm^3. The oocyte-cumulus complexes were obtained by dissection of antral follicles (sized 2–8 mm) of cow ovaries with a safety razor. The manipulations were carried out at 37 °C on a heating table in the medium for collecting oocytes, containing 30 mg of bull serum albumin (Sigma, USA) dissolved in 5 cm^3 of TL HEPES (Minitube, Germany). The oocyte-cumulus complexes were analyzed under a SZ51 stereomicroscope (Olympus, Japan). For the further studies, there were selected oocyte-cumulus complexes with no morphological signs of atresia, having dense cumulus, integral translucent membrane and rounded non-vacuolized homogeneous ooplasm (Fig. 1).

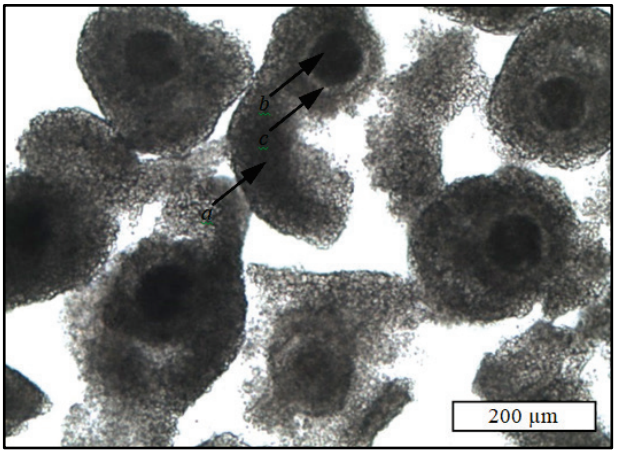

Fig. 1. Oocyte-cumulus complexes of bovines retrieved for maturation: cells of cumulus (*a*), ooplasm (*b*), translucent membrane (*c*)

The oocyte-cumulus complexes of suitable quality were rinsed 6 times in the solution for collecting oocytes and moved to the medium comprising 4.5 cm^3 of TCM 199 (Minitube, Germany), 0.5 cm^3 of estral cow serum, 0.125 M.O. of follicle-stimulating hormone (FSG Super follicule-stimulating hormone drug (Agrobiomed, Russian Federation)), 0.125 IU of luteinizing and follicle-stimulating hormones (50 mm^3 Pluset (Laboratories Calier S.A., Spain)) and 50 mm^3 of antimycotic antibiotic (Sigma, USa). The cultivation was performed in the 4-well plates (Oosafe, USA) in a CO_2-incubator at the temperature of 38.5 °C, 5% O_2 and 6% CO_2 for 22–24 h. To each well of the plate, we introduced 300 mm^3 of the medium and 25 oocyte-cumulus complexes and covered them by a layer of mineral oil (Origio, Denmark).

Preparaton of spermatozoids for fertilization. The bull spermatozoids were prepared for fertilization using the medium for capacitation of spermatozoids (Minitube, Germany) and Origio Gradient Series density gradients (Origio, Denmark). The gradients were adjusted to the room temperature (20–25 °C); the medium for capacitation of spermatozoids was put into a CO_2-incubator in 5% CO_2 conditions at 38.5–39.0 °C for no less than 2 h. The system of gradients was created by a slow overlay on 1 cm^3 of Origio Gradient 40 per 1 cm^3 of Origio Gradient 80 and the sperm was carefully introduced (sperm dose was previously multiplied on a water bath). The formed system was centrifuged for 20 min, the centrifuging force equaling 300 g. The supernatant had been removed, and the sediment was transferred using a sterile tip into a centrifugation test tube with 2 cm^3 of the environment for the preparation and capacitation of spermatozoids, containing 30 mg of bull serum albumin (Sigma, USA), 50 mm^3, antimycotic antibiotic (Sigma, USA), 0.55 mg of sodium pyruvate (Sigma, USA), dissolved in 5 cm^3 of basic solution for capacitation (Minitube, Germany). Then it was subjected to repeated centrifugation for 5 min, the centrifuging force equaling 300 g, and most of the supernatant was removed afterwards. The procedure was repeated twice. After rinsing, the sediment was transferred to the bottom of the centrifugation test tube containing 1 cm^3 of the medium to prepare and capacitate the spermatozoids. The mobile fraction of spermatozoids was obtained using the swim-up method (Parrish et al., 1986). For this purpose, they were incubated for 1 h. This amount of time was enough for the spermatozoids with pathological changes and the dead ones to settle at the bottom of the centrifugation test tube, whereas the mobile spermatozoids had time to ascend to the upper layers of the medium. The capacitation was carried out in the medium for preparation and capacitation by exposure to heparine (Sigma, USA) in the concentration of 20 μg/cm^3 for 4 h in the conditions of CO_2-incubator at the temperature of 38.5 °C with 5% O_2 and 6% CO_2. The capacitated spermatozoids were subjected to centrifugation for 5 min, against the background of 200 g centrifuging force. The supernatant had been removed, and the obtained sediment was supplemented with 1 cm^3 of fertilization medium.

After maturation, the oocytes were cleared from the cumulus cells in 0.1% hyaluronidase solution (Sigma, USA) using a 140 μm-diameter denundation pipette (Cook, USA). Then, we rinsed off the enzyme in 5–6 drops of TL HEPES (Minitube, Germany). For intracytoplasmic spermatozoid injection, we collected only the oocytes with visible first polar body.

The procedure was conducted in a Petri dish with 5 drops of the Universal medium (Origio, Denmark), 5 mm^3 each, placing one oocyte into each, under mineral oil (Origio, Denmark). The spermatozoids were placed into another 3 mm^3 drop of polyvinylpyrrolidone (PVP) (Origio, Denmark), because the significant viscocity of PVP reduces the mobility of spermatozoids (Hyakutake et al., 2015). We carried out the ICSI procedure using an ICSIPulator micromanipulator (Oosafe, USA). To the drop with spermatozoids, an ICSI MIC-9-35 micropipette (Origio, Denmark) was transferred, and one spermatozoid was aspirated in the tail-forward position. The oocyte was positioned so that the first polar body would be in the position for 6 or 12 h and fixated using a K-HPIP-1035 holding micropipette (Cook, USA). Then, the injection pipette was mechanically extruded into the oocyte cytoplasm, and then the oolemma was pierced (Fig. 2). The ooplasm had been carefully aspirated so as to perforate the plasmatic membrane by changing the pressure, driving a part of ooplasm into the injection pipette, and then the spermatozoid was transferred into cytoplasm.

After the ICSI, the oocytes were transferred to thegrowth medium. Artificial activation of bovine oocytes.

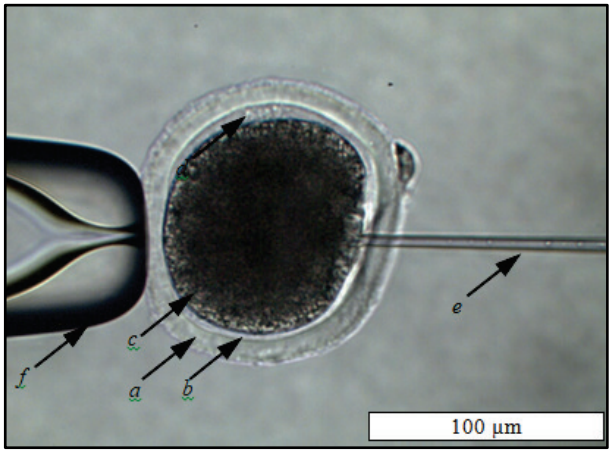

Fig. 2. Intracytoplasmic sperm injection of spermatozoid into bovine oocyte: translucent membrane (*a*), perivitelline space (*b*), oocyte cytoplasm (*c*), polar body (*d*), micropipette for intracytoplasmic sperm injection (*e*), holding micropipette (*f*)

Because the objective of our study was determining the effect of calcium ionophore A23187 (Sigma, USA) on the activation of oocytes, the cells after the ICSI (the total amount was 120) were divided into four groups to confirm the significance of the data; the experiment was repeated three times. The first group was the control (without adding the activator), the second comprised the oocytes activated using 5 μM of calcium ionophore, the third contained those activated using 10 μM, and the fourth those activated by 50 μM. We took into account that fluctuation of Ca^{2+} starts 15–30 minutes after the ICSI (Nakano et al., 1997; Yanagida et al., 2001), which can be associated with delayed release of phospholipase C zeta through a slowed breakdown of the plasmatic membrane of spermatozoids during the intracytoplasmic injection (Dozortsev et al., 1997). The medium was used based on the estimation of 30 mm^3 of the medium per one oocyte. The cultivation was performed at the temperature of 38.5 °C with 5% O_2 and 6% CO_2 and in CO_2-incubator for 15–20 min after the ICSI. After the cultivation, the oocytes were rinsed ione-by-one n two drops of embryo cultivation medium, 30 mm^3 each, and then transferred into the medium for following cultivation (all the manipulations were performed under the layer of mineral oil (Origio, Denmark)). Cells of the first group (control) were transferred to the embryo cultivation medium right away.

Cultivation of embryos. After the activation, the fertilized oocytes were placed in the environment for cultivating bovine embryos, composed of 5 cm^3 of pyruvate (Minitube, Germany), 200 mm^3 of essential aminoacids (Sigma, USA), 50 mm^3 of non-essential aminoacids, 0.5 cm^3 of estral bovine serum, and 50 mm^3 of antimycotic antibiotic (Sigma, USA). The cultivation was performed in well dishes (Oosafe, USA), in 65 mm^3 microdrops (3 embyos in one drop) under a layer of mineral oil (Origio, Denmark), at the temperature of 38.5 °C, with 5% O_2 and 6% CO_2 in CO_2-incubator. The fertilization of oocytes was determined according to the division 2–4-cellular stage 48 h after contacting the spermatozoid. The morula stage was reached on the 3rd day of embryo cultivation, and blastocyte on days 6–8.

The final evaluation of quality of the bovine embryos was performed on the 8th day of the development according to the classification proposed by Bó & Mapletoft (2013), recommended by the International Embryo Transformation Society (IETS) (Rocha et al., 2016). Four groups were designated:

1. Poor quality, embryos that are unsuitable for transplantation or freezing (development stage does not correspond to time, presence of notable changes in size and form of embryo mass, changes in colour and density of individual cells, fragmentation of more than 25% of embryo cells) (Fig. 3d).

2. Stage code 5 (early blastocyte, embryo's cavity is filled by fluid or blastocoels, the general appearance of a signet ring, 70–80% of peryvite-

line space is filled by cellular mass), quality code 1 (embryo has a spherical cellular mass, which is symmetric, and is composed of blastomeres that are same in size, density and colour, corresponds to the expected development stage; can have relatively insignificant irregularities, no less than 85% of the cellular material has undamaged vital embryo weight; the zona pellucida is smooth, has no curves of flat surfaces) (Fig. 3a).

3. Stage code 6 (characterized by a notable differentiation of trophoblast and embryoblast, blastocoel is clearly distinct, embryo takes up the most part of the perivitelline space), quality code 1 (Fig. 3b).

4. Stage code 7 (expanded blastocoel, embryo is increased, densely adjusted to the translucent membrane, which in turn is one third thinner than initially), quality code 1 (Fig. 3c).

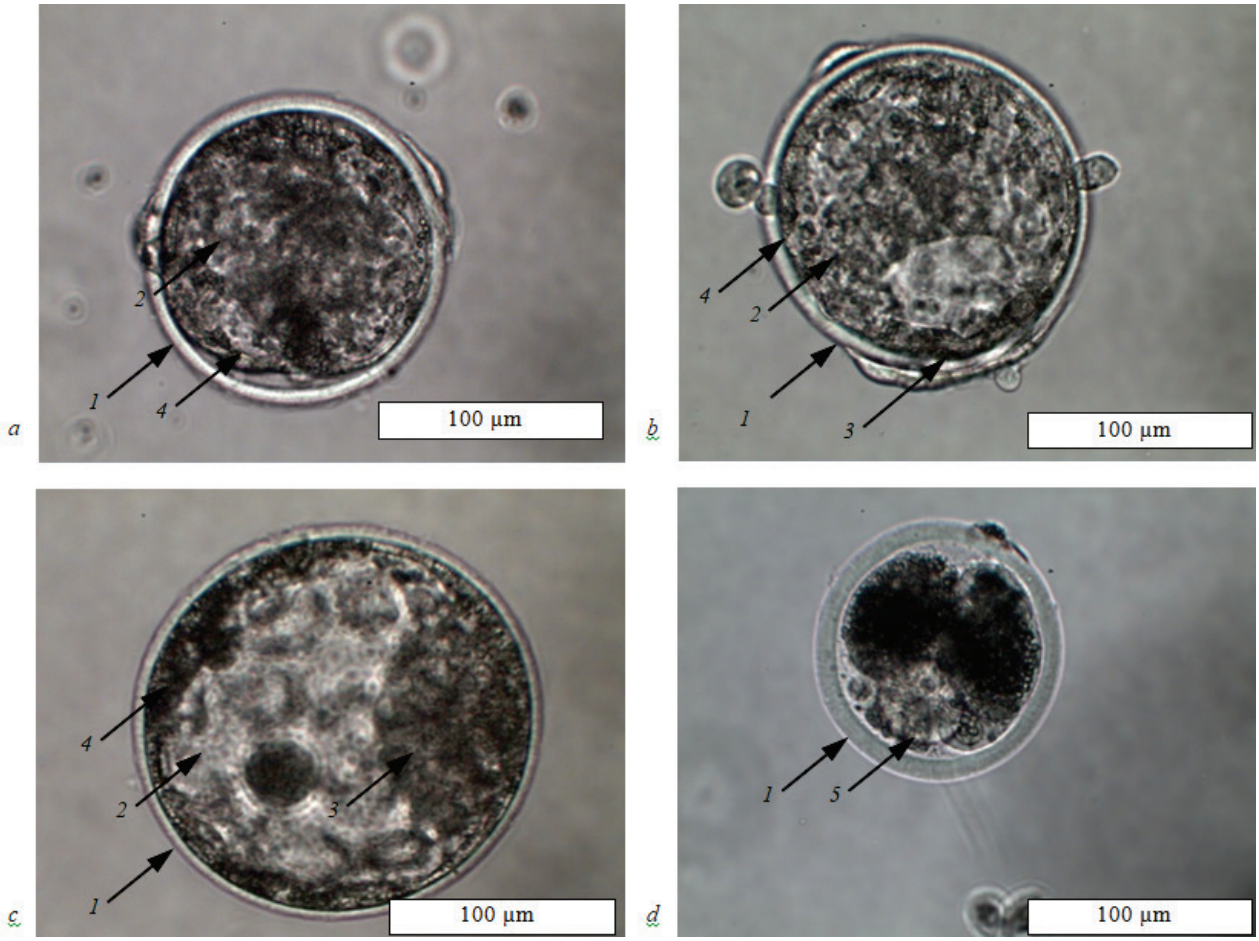

Fig. 3. Bovine embryos at different development stages (day 8): group 1 (stage code 5, quality code 1) (*a*), group 2 (stage code 6, quality code 1) (*b*), group 3 (stage code 7, quality code 1) (*c*), group 4 (unsuitable embryos) (*d*); *1* – zona pellucida, *2* – blastocyst cavity, *3* – inner cell mass, *4* – trophoblast, *5* – fragmentation

The data were statistically analyzed in the ANOVA program, the results in the tables are presented as x ± SE (standard error). To compare the difference of the mean parameters between the control and experimental groups, we used the Tukey test, where the differences are statistically significant at $P < 0.05$ for all the data.

Results

According to the results of the study, the activation of bovine oocytes using calcium ionophorm significantly increased conception (Table 1). However, inferring from the data analysis, we may state that oocyte fertilization did not significantly differ accross the groups with different concentration of the activator.

Therefore, in the control group, the percentage of 2–4-cellular embryos on the 48th h after fertilization accounted for 33.3 ± 4.7, whereas in the groups where the oocytes were activated by 5 and 10 µM ionophorm calcium, we observed 40% higher level of fertilization, and in the group with 50 µM activation, it was 43.0% higher, compared with the control. The obtained data indicate high efficiency of the activation of bovine oocytes using calcium ionophorm.

However, the ratio of fertilized oocytes that reached the blastocyst stage did not significantly differ between the groups. Blastocyst yield in the control group was 38.9 ± 7.9%, while it was 11.1% higher in the group of oocyte activation by 5 µM calcium ionophorm, 9.2% higher using 10 µM, and 14.0% higher using 50 µM. At the same time, the the ratio of blastocysts obtained to oocytes injected significantly differed ($P < 0.05$). In the control group, this parameter equaled 13.3 ± 4.7%, was 23.4% higher using 5 and 10 µM calcium ionophor, and 26.7% higher using 50 µM.

Table 1
Parameters of fertilization and development of bovine embryos after artificial activation of oocytes using calcium ionophore (x ± SE, n = 3)

Concentration of calcium ionophore, µM	Quantity of oocytes	2–4-cellular embryos, %	Blastocytes, %	Blastocytes/ oocytes ratio, %
Without stimulation (control)	30	33.3 ± 4.7[a]	38.9 ± 7.9	13.3 ± 4.7[a]
5	30	73.3 ± 9.4[b]	50.0 ± 0.0	36.7 ± 4.7[b]
10	30	73.3 ± 4.7[b]	48.1 ± 6.4	36.7 ± 4.7[b]
50	30	76.6 ± 9.4[b]	52.9 ± 6.0	40.0 ± 0.0[b]

Note: letters indicate significant differences between groups within column ($P < 0.05$), according to the results of Tukey test.

The evaluation of effect of the artificial activation of oocytes on the development of bovine embryos revealed no significant difference between the groups. The percentage of poor-quality embryos (embryos that are unsuitable for transplantation or freezing) in the control group equaled 20.0 ± 0.0 (we obtained 6 embryos from 30 oocytes used), while after using 5 and 50 µM calcium ionophore, this parameter was higher by

16.6% (11 of 30), and higher by 20.0% (12 of 30) after using 10 μM. We observed no embryos of stage code 5, quality code 1, in the control group, whereas their percentage accounted for 10.0 ± 0.0 (3 of 30) after using 5 and 50 μM of calcium ionophore, and 6.7 ± 4.7 (2 of 30) after using 10 μM. Also, we saw no significant difference in the percentage of embryos of stage code 6, quality code 1, across the groups: 20.0 ± 4.7% (6 of 30) – the control, 20.0 ± 0.0% (6 of 30) using 5 μM calcium ionophore and 23.3 ± 4.7% (7 of 30) using 10 and 50 μM of the activator. We observed no embryos of stage code 7, quality code 1, in the control group, while after using the activator, their percentage equaled 6.7 ± 4.7 (2 of 30) in all the groups.

The results suggest that calcium ionophore has a significant effect on the fertilization of bovine oocytes and the yield of blastocytes in relation to the gametes used. At the same time, this activator did not affect the further development of embryos, as indicated by the parameters of the development of blastocysts from 2–4-cellular embryos and the evaluation of their quality.

Discussion

After natural fertilization, a mature oocyte is activated right after the fusion with spermatozoid (Whitaker & Patel, 1990; Ferrer-Buitrago et al., 2018). This mechanism is a trigger of the fertilization. Further fluctuations of intracytoplasmic concentration of calcium is the result of release of phospholipase C zeta from the spermatozoid head into the ooplasm, which plays key role in the further development of the embryo (Parrington et al., 2007; Kashir et al., 2010). Increase of Ca^{2+} in mammals occurs periodically in the form of long fluctuations, which starts after the spermatozoid fuses with the oocyte (Lawrence et al., 1997; Stricker, 1999) and stops around the first interphase, as it takes place in mice (Marangos et al., 2003), or as the first mitotic cellular cycle approaches, as it occurs in bovine cattle (Nakada et al., 1995). Those fluctuations are enough for the completion of the oocyte activation, which initiates the blocking of polysperm (exocytosis of the material of cortical granules) (Wong & Wessel, 2005), recovery of meiosis and transfer to the embryonal cellular cycle, and also changes in the expression of genes and their protein products in the zygote and early embryo (Ozil & Huneau, 2001; Ducibella et al., 2002). However, this order of events is typical for natural fertilization of coculturing of gametes. After the ICSI, intracytoplasmic increase in Ca^{2+} occurred directly during the spermatozoid injection into oocyte cytoplasm, the source of calcium in this case being the culture medium (Tesarik et al., 2000).

The supposition that oocyte activation is a chemical process that includes changes in the concentration of ions inside gametes of females was for the first time proposed by Loeb (1899) in 1898. The key step in confirming this theory was the studies in which oocytes were treated with calcium ionophore A23187 (Steinhardt & Epel, 1974), which caused their activation. This suggests that artificial increase in Ca^{2+} inside oocyte induces processes associated with its activation (Steinhardt et al., 1974). Later, it was revealed that Ca^{2+} increase in female gametes accompanies the fertilization (Ridgway et al., 1977; Miyazaki & Ito, 2006). Blocking of this Ca^{2+} increase using chelate agents inhibits oocyte activation (Whitaker & Steinhardt, 1982; Yang et al., 2018). However, many of those studies were for this time performed on gametes of sea urchins. Nonetheless, it was determined that Ca^{2+} definitely acts as a trigger of activation of oocytes in practically all species of animals (Stricker, 1999; Whitaker, 2006).

Despite the fact that calcium ionophore A23187 was for the first time used for oocyte activation in 1974 (Steinhardt & Epel, 1974), its use still remains one of the most effective methods of oocyte activation, which is broadly used in clinical practice. It has a certain lipophilicity and can neutralize the positive charge of Ca^{2+} (Ducibella & Fissore, 2008), and also promotes release of intracellular Ca^{2+} reserves, and improves the ingress of extracellular Ca^{2+} (Alberio et al., 2001), since it can move extracellular Ca^{2+} against the concentration gradient, increasing its concentration in ooplasm (Ducibella & Fissore, 2008). Moreover, it promotes the activation of oocytes, improves the fertilization rates, formation of blastocysts, implantation of embryos and live birth (Murugesu et al., 2017). Moreover, the activation using calcium ionophore A23187 has no effect on the percentage of early embryonic mortality, blastocyst aneuploidy, pregnancy period, and birth weight (Zhang et al., 2023), and does not increase the likelihood of meiosis II errors (Capalbo et al., 2016) and the frequency of inherent developmental abnormalities (Miller et al., 2016; Mateizel et al., 2018).

There are a great number of studies on artificial activation of bovine oocytes using assisted reproductive technologies. However, their results are still contradictory. In particular, the reports are hard to compare because of differences in the results obtained using the same methods of activation in various studies, which can be explained by differences in the cultivation conditions after oocyte activation, different ICSI techniques and differences in qualification of embryologists who performed fertilization (Kovpak et al., 2022). Nonetheless, most researchers agree that the ICSI is low-effective for bovines without the activation of bovine oocyte. In general, Rho et al. (1998) and Chung et al. (2000) indicate absence of embryos at the stage that is sufficient for successful transplantation if oocytes had not been activated after the ICSI. Therefore, there should be comparison of the activation of bovine oocytes induced by various concentrations of calcium ionophore A23187 with further studies of its effect on preimplantation embryonic development and design of protocols adjusted for the territory of Ukraine is justified.

Over the process of literature analysis, we found that most researchers activated bovine oocytes using 5 μM of calcium ionophore, which had a positive effect (Suttner et al., 2000; Wang et al., 2008; Acar & Bastan, 2011). Wang et al. (2008) indicate that the use of calcium ionophore A23187 (5 μM for 5 min) for the activation of bovine oocytes causes increase in the fragmentation ($P < 0.05$) within 11.3–23.7% (3.9–5.3% without stimulation). In turn, according to Suttner et al. (2000), oocyte cultivation after ICSI with 5 μM of calcium ionophore in 400 μL of PBS for 10 min resulted in 79.6% level of early division, which was much higher, compared with the other study protocols ($P < 0.01$). Acar & Bastan (2011) suggest that the rates of fragmentation of oocytes, activated by 5 μM of calcium ionophore, were 44.8% (without stimulaton – 26.8%, $P < 0.001$), the percentage of morules accounted for 17.2 (without stimulation – 9.2) and the frequency of blastocyst formation equaled 11.6% (without stimulation – 3.2%). However, Chen & Seidel (1997) used 50 μM of calcium ionophore A23187 and observed significantly higher ($P < 0.05$) fragmentation percentage, equaling 47% (33/70), 25% (17/68) compared with the group with no stimulation, and higher number of blastocysts (16%), compared with the control (6%, $P < 0.05$). At the same time, Keefer et al. (1990) indicate that after the activation with 1 μM of calcium ionophore A23187, oocytes divided normally following spermatozoid-injection fertilization. Therefore, after 5-min exposure to 1 μM A23187, nuclear decondensation of spermatozoids occured 3 h later in 37% (inactivated – 0%), and 48 h after the fragmentation up to the stage of 2–4 cells in 28%, 6/21 (inactivated – 4%).

In our study, we observed a significantly higher level of fertilization after activating bovine oocytes with calcium ionophore, compared with the group with no activation ($P < 0.05$), which is consistent with the results of other researchers. However, no significant differences were seen in the effects of various concentrations of the activator on the percentage of obtained 2–4-cellular embryos, this parameter fluctuating within 73.3–76.6%, which correlates with the data of Suttner et al. (2000), though it was significantly higher than in the studies by Wang et al. (2008), Chen & Seidel (1997), Acar & Bastan (2011) and Keefer et al. (1990). In addition, we observed a significant difference in the ratio of the amount of blastocysts obtained to oocytes used; this indicator equaled 13.3 ± 4.7% (4/30) without the activation, increased up to 36.7 ± 4.7% (11/30) after using 5 and 10 μM of calcium ionophor, and up to 40.0 ± 0.0% (12/30) after using 50 μM. It has to be noted that the parameters of the development of blastocysts from 2–4-cellular embyos and evaluation of their quality according to the activation of oocytes by calcium ionophore were not significantly different from the control group.

Thus, the results suggest the high efficacy of calcium ionophore on the activation of bovine oocytes after intracytoplasmic spermatozoid injection, facilitating the production of a larger amount of embryos suitable for transplantation or freezing. The presented protocols of the activation and cultivation of oocytes can be valuable for programs of transplantation of *in-vitro*-produced embyos because they allow fertilization using not only gametes of suitable quality, but also low-quality sperm from high-value sires and cryoconserved oocytes.

Conclusion

As of now, there are factors which stand in the way of practical application of the methods of intracytoplasmic spermatozoid injection in animal farming. Therefore, insufficient activation of bovine oocytes during spermatozoid microinjection leads to low percentage of fertilization and therefore a decrease in the ratio of blastocysts obtained to oocytes used. However, the use of calcium ionophore for artificial activation of bovine oocytes after intracytoplasmic spermatozoid injection can solve this problem.

The study of the effect of various concentrations of calcium ionophore on fertilization revealed significantly higher percentage of 2–4-cellular embryos on the 48th h after intracytoplasmic spermatozoid injection in the groups where oocytes had been activated, compared with the control (with no activation). This parameter accounted for 73.3 ± 9.4% and 73.3 ± 4.7% after using 5 and 10 μM of calcium ionophore, respectively, and 76.6 ± 9.4% (33.3 ± 4.7% in the control) in the group with addition of 50 μM. This in turn significantly increased the percentage of obtained blastocysts to oocytes used, compared with the control. This parameter equaled 36.7 ± 4.7% in the groups where 5 and 10 μM of calcium ionophore was used, and 40.0 ± 0.0% in 50 μM group (13.3 ± 4.7% in the control). Evaluation of the quality of embryos revealed no significant differences in the parameters, though it has to be noted that the calcium-ionophore activation of bovine oocytes after intracytoplasmic spermatozoid injection resulted in 11 embryos suitable for transplantation or freezing from 30 used egg cells in the group where 5 and 10 μM of calcium ionophor had been used, 12 from 30 after using 50 μM of the activator, against 6 out of 30 in the control group. The results of the study and the data obtained by other researchers demonstrate the benefits of introduction of artificial activation of bovine oocytes after intracytoplasmic spermatozoid injection of calcium ionophore to the standard protocols.

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