BOVINE AND SWINE PARTHENOTS GENERATING THROUGH ELECTRICAL STIMULATION OF THE OOCYTES

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Abstract

Electrical stimulation is an alternative to chemical activation to induce $Ca2^{+}$ influx, responsible for the formation of pores in the cellular membrane. In order to activate the oocytes, electrical stimulation (E.S.) was performed on 30 oocytes derived from gilts (L1), sows (L2), heifers (L3) and cows (L4). We considered that the stage of development of four cells is eloquent for certifying the ES's division triggering and the results we are considering only refer to these parthenots. Following application of ES, oocyte activation occurred as follows: 6.6% at L1, 16.6% at L2, 20% at L3 and 46.6% at L4. It is obvious the higher maturation rate of oocytes from adult females as compared to young females (16.6% in sows versus 6.6% in gilts and 46.6% in cows versus 20% in heifers). The method of electrical stimulation of oocytes in the fusion chamber used in this paper is effective for activating the division in both bovine and swine oocytes. Activation of oocyte division following electrical stimulation is clearly superior when using oocytes from adult females. The electrical stimulation method used generated the upper division activation in cattle compared with the results obtained using swine oocytes.

Keywords*: oocytes electrical activation, ART technology*

Introduction

Activation of the oocyte during fertilization is caused by intracellular calcium oscillations whitch is triggered by the spermatozoa entry, after this, a series of events makes fertilization complete such as: inactivation of maturation promoting factor (MPF) and of mitogen activated protein kinase that leads to resumption and completion of meiosis, DNA synthesis and pronuclei formation (Paffoni et al., 2008).

Intracellular calcium increase in the oocyte can be induce without a spermatozoa, by using activating agents such as: ethanol, Ca^{++} ionophores and electroporation (Paffoni et al., 2008).

Artificial oocyte activation is used in assisted reproduction laboratories as a step in obtaining parthenogenetic embryos - source of parthenogenetic embryonic stem (pES) cells. These pES cells might serve as a source of tissue for transplantation (Kim et al., 2007). Parthenogenesis consists in the growth and development of embryos from oocytes that have not been fertilized by spermatozoa (Bevacqua et al., 2011). Also during some protocols of nuclear transfer (NT) enucleated *in vitro* maturated metaphase II oocytes and microinjected with the donor cell into the perivitelline space, are activated by electrical pulses. As donor cell can be used cumulus, oviduct, skin, liver cells, blastomeres, fibroblast, adipocytes and other types of cells (Lai and Prather, 2003).

By comparing the degradation rate following parthenogenetic activation with that obtainded after IVF, Cevik et al. (2009) did not notice significant differences, indicating that the activation of bovine oocytes by electrical stimulation and chemical agents yields good results, and the culture medium can support parthenogenetic development. In swine, oocyte activation as measured by the presence of pronucleus varies from 22% to 74%. The DC voltage field pulses cause temporary formation of pores in the plasma membrane, thus allowing extracellular and

intracellular exchange of ions and molecules. The number of blastocyst cells is a good indicator of embryo quality.

The purpose of this paper was to observe how oocytes from cattle and swine at different reproductive stages respond to the electrical stimuli used to activate cell division.

Materials and methods

Oocytes were obtained from ovaries from heifer, cows, gilt and sows. They were transported in 0.9% NaCl solution in isothermal bags at a temperature of 25-38°C. The ovaries were brought from the Smithfield slaughterhouse in Timisoara and from Macea, Arad County, which is 80.5 km (1h and 9 min) to the assisted reproduction lab of CLC-HC. For harvesting oocytes, the method of suction and cultivation of category I oocytes was chosen.

In vitro maturation was performed in 400 µl of TCM 199 medium supplemented with 10% ECS and coated with mineral oil at 38.5°C, 5% CO_2 for 44 hours for sow oocytes and 24 hours for bovine. For denudation, 0.1% hyaluronidase was used.

The extraction of the second polar body was performed using the Axiovert 40 CFL Narishige micromanipulation system, equipped with a NIKON reversed phase contrast microscope. Enucleated oocytes were placed in the culture medium in the incubator immediately after enucleation to allow the membrane and the cytoskeleton to recover rapidly after enucleation procedures.

The next step is the transfer of cumulus cells into the enucleated oocytes. Cumulus cells used as donor cells were not treated to reach a stage of the cell cycle, after some authors were G0 or G2/ M after other authors (Lai and Prather, 2003). For balancing, they are left for 10 seconds in the electrofusion medium.

The electrofusion process was performed using the Electro Cellfusion CFA 500 (Krüss, GmbH).

The device was set as follows: AC voltage: 0, AC duration: 0, AC post fusion time: 0, DC voltage: 160 V, DC pulse lenght: 30 µsec, number of pulses: 3, field power: 1.6 kV/ cm, fusion camera: BTX Microslide model 450-1 (1mm gap).

The electrofusion medium is composed of a 0.3 M medium consisting of 0.5 mM HEPES, 0.01% BSA, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$.

The reconstitued oocytes are then placed in the fusion chamber and covered with fusion medium. Using a pipette, the reconstitued oocytes were manually arranged so that the contact surface between the cytoplasm and the donor cell became parallel with the electrodes. Then the electric shock was applied.

The reconstitued oocytes were transferred to the maturation medium with TCM 199 and 15% ECS and incubated at 38.5°C, 5% CO_2 and constant humidity. Production of the division was checked every four hours, and the medium was changed every 24 hours.

Results and discussions

The results obtained following the use of electroactivative technique of bovine and swine oocytes are inserted in table 1 and shown in figure 1 and 2.

Table 1.

Results obtained after oocyte activation following electrostimulation

	Oocytes that started the cellular division	
	n	**%**
Gilts	2	6.6
Sows	5	16.6
Heifers	6	20
Cows	14	46.6

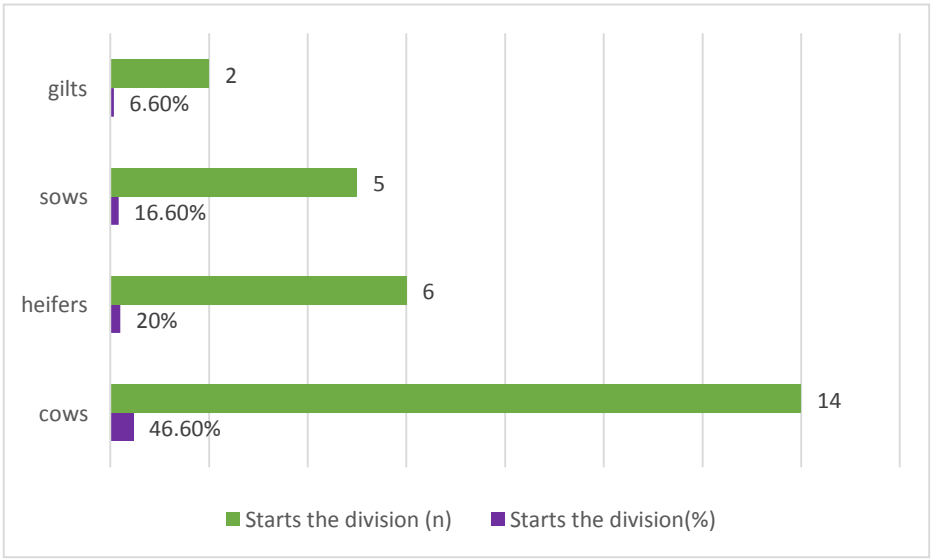

Figure 1. Cattle and swine oocytes activation following electrostimulation

For oocyte activation, electric stimulation was performed on 30 *in vitro* maturated oocytes, from gilts (L1), sows (L2), heifers (L3) and cows (L4). We considered that the stage of four cells is eloquent for certifying the ES's division triggering and the results we are considering only refer to these parthenots.

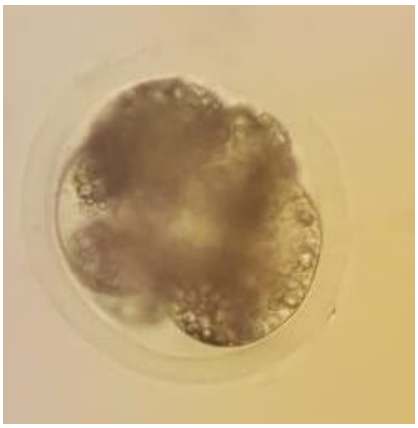

Figure 2. Swine parthenot in the four-cell stage

Observations were made at regular four-hour intervarls, the divisions emerging after 24 hours.

Following application of ES, oocyte activation occured as follows: 6.6% at L1, 16.6% at L2, 20% at L3 and 46.6% at L4. It is evident the higher maturation rate of oocytes from adult females as compared to young females (16.6% in sows versus 6.6% in gilts and 46.6% in cows versus 20% in heifers). It is supposed that this may be due to the increased availability of oocytes from adult females to go through consecutive SE divisions, probably through the existence of already mature cell cycle paths in correlation with the generative stimuli of the division. It is widely accepted that the cytoplasm of oocytes contains the necessary information for nuclear reprogramming, thus enabling the cell division to be triggered following electrostimulation.

Different biology of sexual cycles between bovine and swine species has an impact on the superior percentage of bovine oocytes that starts the division (both in adult and young female). Figure 1 clearly reveals these differences and allows us to observe the division of oocytes.

In 2017, Keller et al. obtain 14.63% zygotes in the four cells stage consecutive ICSI in cattle and in 2016, Godja et al. signals ICSI generation of 30% divisions attested by expressing the two pronuclei.

Consistent with the present results, previous studies have confirmed that parthenogenetic bovine blastocyst have a total number of cells significantly lower than IVF blastocyst.

Milazzotto et al. (2008) have demonstrated that despite the fact that there is no difference between electrical stimulation and chemical activation on blastocysts production rates, electrical activation has determined blastocysts with a higher percentage of viable cells.

During fertilization, oocyte activation is induced by the release of intracellular calcium after binding of sperm to the plasma membrane of oocytes. The meiotic resumption occurs because of the transient calcium oscillations. Thus, differences in blastocyst rates in this study can be explained by different calcium oscillation models that have been promoted through various treatments and by the addition of BSA as an activating attenuation agent because albumin has important calcium binding properties. Intracellular calcium oscillations are known to mediate cellular functions, such as gene expression and cell cycle regulation.

Rizos et al. (2002) demonstrated that the embryo culture medium plays a crucial role in determining the quality of the blastocyst. The number of blastocyst cells was higher when the

activated oocytes were co-cultured in TCM 199 with bovine oviduct epithelial cells or grown in SOF with BSA. When the number of viable cells was evaluated, the first group had better results. Ozil and Huneau (2001) reported changes in calcium influx during oocyte activation affecting the development of post-implant embryo in rabbits due to interference in the epigenetic reprogramming of the zygote genome. These epigenetic abnormalities are transmitted through blastomere divisions and lead to changes in gene expression patterns.

It is widely accepted that this influx of calcium during oocyte activation may interfere with the expression of antiapoptotic and proapoptotic genes in preimplantated embryos.

Wu et al. (2017) investigating the survival and activation of the oocytes vitrified before and after electrostimulation, have demonstrated that the oocytes vitrified at 4-5 hours after electrostimulation generated a satisfactory survival rate as well as the pronuclei formation.

Li et al. (2017) studied th *in vitro* growth of oocytes activated by gene stimulation and then treated with various concentrations of AZD5438, inhibitor of cyclin-dependent kinases 1,2 and 9. The results obtained demonstrate that the electrical activation of swine oocytes in combination with AZD5438 treatment, lead to an increased rate of blastocyst formation in parthenogenetic activation as well as in somatic cell nuclear transfer experiments.

Following the ability to develop oocytes activated by an electric stimulus and treated with anisomicin, Zhang et al. (2017) have shown that using this method results in an increased percentage of blastocysts.

Conclusions

The method of electrical stimulation of oocytes in the fusion chamber used in this paper is effective for activating the division in both bovine and swine oocytes.

Activation of oocyte division following electrical stimulation is clearly superior when using oocytes from adult females.

The electrical stimulation method used generated the upper division activation in cattle compared with the results obtained using swine oocytes.

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