

Parthenogenetic development of rabbit oocytes after electrical stimulation

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ABSTRACT: The aim of this study was to determine the effect of electric pulses on the structural and functional condition of rabbit oocytes. The New Zealand White female rabbits at 3–5 months of age and at 3–4 kg body weight served as oocyte donors. Oocytes after flushing from the oviducts were placed between two electrodes in an electroporation chamber which was filled with a dielectric solution. Following a short incubation in B2 medium, oocytes were subjected to an electric pulse released by an electrical pulse generator. Oocytes were then incubated in 500 µl of B2 medium supplemented with 20% foetal calf serum (FCS) at 38°C in an atmosphere of 5% CO₂ in air. Oocytes were cultured until the morula/blastocyst stage (approx. 72 h). The experiment was conducted using 430 oocytes obtained *post mortem*. *In vitro* cultured oocytes not subjected to an electric pulse were the control. Each group was subdivided into replications according to electric current intensity. The analysis of experimental variants shows that in the first variant all embryos developed to the morula stage but only 10% of them continued to develop to the blastocyst stage. In the second variant we observed that 5–10% of oocytes developed to the blastocyst stage after treatment with 2.0 and 2.5 kV/cm pulse but in the group of 1.0 kV/cm pulse 35% of oocytes developed only to the 2–12 b stage. In the third variant only 1 oocyte (5%) continued to develop to the blastocyst stage, but in the fourth variant oocyte development stopped at the morula stage. In the fifth variant, called an “extreme” one, oocytes stopped to develop at the stage of 2–12 b (about 25%) and the percentage of degenerated oocytes dramatically increased (about 60%).

Keywords: rabbit oocytes; electric pulses; *in vitro* culture

In the cloning procedure using cell nuclear transplantation, cell donors of exogenous nuclei are *in vitro* or *in vivo* mature oocytes arrested at the metaphase of the second meiotic division.

In the first stage of cloning, metaphase chromosomes are removed microsurgically (enucleated) and an exogenous embryonic or somatic nucleus is inserted in their place into enucleated oocytes (cytoplasm). The most common procedure for reconstructing enucleated oocytes is a fusion with the whole embryo or somatic cell. The fusion can be induced by a variety of physicochemical methods. Then the fusion of these two structures has to be initiated.

There are two methods of fusion in an electric field. In the direct method, the cell fusion takes

place directly in an electric field. In the indirect method, the fusion in an electric field is performed between oocytes and cytoplasts containing a foreign cell nucleus that has been introduced using other fusiogenic factors, e.g. polyethylene glycol or inactivated Sendai virus. However, this method is not very common. Ozil (1990), who performed close observations on the activation of rabbit oocytes, formation of pronuclei and further development of the obtained parthenogenetic embryos, reported different degrees of activation and only the oocytes that were properly activated stood a chance of further development.

A very important aspect of somatic cloning is artificial activation of reconstructed oocytes. For

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each animal species, activation protocols have been developed which specify an activator as well as the appropriate moment when the activator should be applied.

After the introduction of an exogenous cell nucleus into the enucleated oocyte, the reconstructed oocytes are subjected to artificial activation to unblock the arrested cell cycle of the oocyte at metaphase II. In the activation we can use different physical and chemical factors. Among the most frequent physical methods are direct current pulses with defined parameters, while chemical methods include ethanol, ionomycin, and calcium ionophore A23187 (Cibelli et al., 1998; Wells et al., 1999; Dinnyes et al., 2001).

Rabbit oocytes can be activated over a broad range of electric field intensity. However, pronuclei are formed and further development to the blastocyst stage occurred only in 35% of cases (Onodera and Tsunoda, 1989).

The effect of electric pulses on the oocyte structure, such as fusion between somatic cell and enucleated oocyte or activation, can cause an apoptotic process, and then the cloned embryos can lose the ability to achieve full development. The possibility of such abnormalities appearing in oocytes subjected to an electric pulse prompted studies aimed at explaining whether low cloning efficiency may spread from developmental anomalies of oocytes subjected to an electric pulse.

MATERIAL AND METHODS

Preparation of oocyte-donor does

Oocyte donors were New Zealand White (NZW) does aged 3–5 months and weighing 3–4 kg. To induce superovulation, females were treated hormonally by intramuscular administration of 100 i.u. of PMSG (pregnant mare serum gonadotropin) (Serogonadotropina, Biowet, Poland). After 72 h, females were injected intravenously with 100 i.u. of hCG (human chorionic gonadotropin) (Biogonadyl Biomed, Poland).

Collection of oocytes

Twenty-four hours after hCG administration, females were sacrificed and oocytes were flushed from the prepared oviducts of donors using buff-

ered PBS solution (37–38°C) supplemented with bovine serum albumin (Sigma, USA) at a rate of 0.4 g/100 ml. Oocytes were selected using a stereoscopic microscope based on the presence of the first polar body.

Exposure of rabbit oocytes to electric field

Oocytes were placed between two electrodes in an electroporation chamber filled with an isotonic dielectric solution (0.3M mannitol supplemented with 0.01mM MgSO₄, 0.1mM CaCl₂) and fatty acid-free bovine serum albumin (FAF/BSA). After a short incubation period in the electroactivation medium, oocytes were subjected to an electric pulse released by an electrical pulse generator (BTX, San Diego, CA).

Physical parameters of electroporation

In the experiment, five variants differing in the number and duration of pulses were used. Direct current intensity was the same for all the variants. The experimental variants were: **variant I** – 1 pulse of 30 µsec (1.0 kV/cm; 1.5 kV/cm; 2.0 kV/cm; 2.5 kV/cm); **variant II** – 1 pulse of 60 µsec; **variant III** – 3 pulses of 30 µsec; **variant IV** – 3 pulses of 60 µsec; **variant V (extreme)** – 3 pulses of 60 µsec (3.0 kV/cm; 3.5 kV/cm; 4.0 kV/cm; 4.5 kV/cm).

In vitro culture of rabbit oocytes subjected to electroporation

Oocytes subjected to the electric field were incubated in 500 µl of B2 medium (INRA Laboratoire C.C.D., France) for 24 hours. After this time, oocytes were placed in the same medium supplemented with 20% foetal bovine serum (FBS) at 38°C in an atmosphere of 5% CO₂ in air. Oocytes were cultured until the morula/blastocyst stage (72 h).

Statistical analysis

The results were analysed statistically using the analysis of variance. The GLM procedure of the SAS packet and Duncan's multiple range test were used for the calculations.

RESULTS

Assessment of *in vitro* development of rabbit oocytes subjected to electric field

In our experiment we used 430 oocytes. Non-treated 30 oocytes were the control.

Analysis of the experimental variants shows that in the first variant, parthenogenetic oocytes developed to the morula stage after treatment with 1.0 kV/cm, 1.5 kV/cm, 2.0 kV/cm pulse, but

only 10% of oocytes continued to develop to the blastocyst stage after treatment with 2.5 kV/cm pulse.

In the second variant, with a longer duration of the electric pulse we observed that also 5–10% of oocytes developed to the blastocyst stage after treatment with 2.0 and 2.5 kV/cm pulse. In the group of 1.0 kV/cm pulse 35% of oocytes developed only to the 2–12 b stage.

In the third variant in the group of 1.5 kV/cm pulse only 1 oocyte (5%) continued to develop to

Table 1. Evaluation of *in vitro* development of rabbit oocytes in an electric field of varying electrostatic intensity after 72 h of culture

Field strength	Number of oocytes	Stage of development			Number of fragmented oocytes (%)	Number of degenerated oocytes (%)
		2–12 b (%)	morula (%)	blastocyst (%)		
1 pulse, 30 μ sec						
1.0 kV/cm	20	9 (45)	3 (15)	0	4 (20)	4 (20)
1.5 kV/cm	20	8 (40)	4 (20)	0	2 (10)	6 (30)
2.0 kV/cm	20	9 (45)	1 (5)	0	2 (10)	8 (40)
2.5 kV/cm	20	5 (25)	5 (25)	2 (10)	4 (20)	4 (20)
Control	30	3 (10)	2 (6, 7)	0	10 (33, 3)	15 (50)
1 pulse, 60 μ sec						
1.0 kV/cm	20	7 (35)	0	0	6 (30)	7 (35)
1.5 kV/cm	20	7 (35)	4 (20)	0	0	9 (45)
2.0 kV/cm	20	6 (30)	1 (5)	1 (5)	4 (20)	8 (40)
2.5 kV/cm	20	10 (50)	2 (10)	2 (10)	1 (5)	5 (25)
Control	30	3 (10)	2 (6, 7)	0	10 (33, 3)	15 (50)
3 pulse, 30 μ sec						
1.0 kV/cm	20	11 (55)	2 (10)	0	2 (10)	5 (25)
1.5 kV/cm	20	7 (35)	2 (10)	1 (5)	2 (10)	8 (40)
2.0 kV/cm	20	8 (40)	0	0	2 (10)	10 (50)
2.5 kV/cm	20	7 (35)	1 (5)	0	3 (15)	9 (45)
Control	30	3 (10)	2 (6, 7)	0	10 (33, 3)	15 (50)
3 pulse, 60 μ sec						
1.0 kV/cm	20	2 (10)	2 (10)	0	6 (30)	10 (50)
1.5 kV/cm	20	1 (5)	2 (10)	0	9 (45)	8 (40)
2.0 kV/cm	20	1 (5)	1 (5)	0	6 (30)	12 (60)
2.5 kV/cm	20	1 (5)	1 (5)	0	10 (50)	8 (40)
Control	30	3 (10)	2 (6, 7)	0	10 (33, 3)	15 (50)
3 pulse, 60 μ sec (extreme variant)						
3.0 kV/cm	20	5 (25)	0	0	2 (10)	13 (65)
3.5 kV/cm	20	4 (20)	0	0	5 (25)	11 (55)
4.0 kV/cm	20	5 (25)	0	0	5 (25)	10 (50)
4.5 kV/cm	20	5 (25)	0	0	3 (15)	12 (60)
Control	30	3 (10)	2 (6, 7)	0	10 (33, 3)	15 (50)

the blastocyst stage, but in the fourth variant oocyte development stopped at the morula stage.

In the extreme fifth variant, called an “extreme” one, oocytes stopped to develop at the stage of 2 to 12 b (about 25%) and the percentage of degenerated oocytes increased dramatically (about 60%).

The analysis of all experimental variants indicates gradual and slight increases in the percentage of degenerating oocytes (Table 1).

DISCUSSION

Research on the activation of oocytes using an electric current was started in mice by Tarkowski et al. (1970). Over the next years, these studies benefited from advances in cloning technique. So far most studies have involved mouse oocytes (Tarkowski et al., 1970; Tarkowski, 1971; Collas et al., 1989; Onodera and Tsunoda, 1989), and also rabbit oocytes (Onodera and Tsunoda, 1989; Ozil, 1990; Modliński and Smorąg, 1991; Escriba and Garcia-Ximenez, 1999, 2000a, 2000b) and pig oocytes (Prochazka et al., 1992; Liu and Moor, 1997; Gruppen et al., 1999). The above authors tried to identify the optimum electric field parameters for effective development of oocytes in different species.

Electric pulses administered at precise intervals are known to induce oocyte activation (Collas et al., 1989; Ozil, 1990) and may be a convenient method for regulation of intracellular Ca^{2+} . In a study by Collas et al. (1989) investigating a variety of factors affecting electrical activation in mouse oocytes, no specific field strength or pulse duration was found to be optimal for activation. The activation rate was much more influenced by the age of oocyte and number of pulses than by pulse parameters. Similar observations have been made for the rabbit. The age of the oocyte and the use of multiple pulses greatly enhance the activation response (Collas and Robl, 1990). Furthermore, properly activated rabbit oocytes are capable of developing to the blastocyst stage *in vitro* (Onodera and Tsunoda, 1989; Ozil, 1990). It is well known in other cells that an electrical shock causes a reversible breakdown of the plasma membrane and, consequently, a dramatic increase in conductivity and permeability, favouring an exchange of intracellular and extracellular components by diffusion (Zimmermann and Vienken, 1982). This temporary destabilization leads to the formation of temporary

pores in the plasma membrane, which allows for the exchange of ions from inside and outside the membrane and for the flow of macromolecules. The size of these pores depends on several factors such as changes in the membrane potential induced by electric pulse, pulse duration, concentration of ions in the electroactivation medium, and the type of cells (Machaty and Prather, 1998). Observations of this kind were made on different species of animals (Tarkowski et al., 1970; Prather et al., 1987; Stice and Robl, 1988; Smith and Wilmut, 1989; Collas and Robl, 1990).

These authors concluded that the rate of activation in oocytes was strictly correlated with the concentration of calcium ions in the medium, and did not necessarily depend on activation using electric pulses. Ozil (1990) analyzed the parthenogenetic development of rabbit oocytes using different electric field parameters. He observed that the cyclically recurring electric stimulation of very long duration (as long as 90 min) resulted in oscillations in the concentration of calcium ions similar to those that occur during fertilization, which, in turn, stimulate the development of embryos to 10–11 days of gestation. Almost all diploid embryos developed to the blastocyst stage. Approximately 30% of them were capable of implantation, 50% of which survived to day 11 of gestation.

Studies on the activation of rabbit oocytes revealed that when 4 short-duration (30 μ sec) pulses were applied, a considerably lower percentage of activated oocytes (58%) was obtained compared to 8 and 12 pulses, for which the percentage of activated oocytes was 88 and 94%, respectively. For longer pulse duration (60 μ sec), the number of pulses applied was observed to have no effect on the activation percentage (91% and 100%). For shorter pulse duration (30 μ sec), the percentage of correctly activated oocytes (PB1 + PB2 and divided oocytes) increased markedly when the number of pulses was increased (Escriba and Garcia-Ximenez, 1999).

In their studies on the activation of porcine and bovine oocytes, Liu and Moor (1997) and Escriba and Garcia-Ximenez (1999) observed both the number of pulses and different intervals between pulses to have a marked effect on the division and developmental capacity of the activated oocytes. The degree of division checked 24 h after activation was lower for 1 pulse or 3 successive pulses applied at 1-sec intervals than for 3 pulses applied at 5-min intervals. In summary, these researchers

concluded that the use of multiple pulses at appropriate intervals increased the developmental potential of activated oocytes.

Similar studies were performed by Hyun et al. (2003), who observed the parthenogenetic development of porcine oocytes activated with an electric field (1 pulse of 30 μ sec) of varying intensity, using 7% ethanol and combining these two activation factors. They concluded that oocytes developed better when the first factor, or a combination of two factors (electric and chemical), was used than when only the chemical factor was used.

In the present study, the *in vitro* parthenogenetic development of rabbit oocytes treated by an electric field was analyzed in five variants.

The analysis of experimental variants shows that in the first variant, after treatment with electric pulse of different parameters 5–25% of oocytes developed to the morula stage but only 10% of oocytes continued to develop to the blastocyst stage in the group of 2.5 kV/cm pulse. In the second variant with 1 impulse and 60 μ sec we observed that 5–20% developed to the morula stage and also about 10% of oocytes continued to develop to the blastocyst stage after treatment with 2.0 and 2.5 kV/cm pulse. In this variant in the group of 1.0 kV/cm pulse 35% of oocytes developed only to the 2–12 b stage. In the group of 3 impulses and 30 μ sec (third variant) 5–10% of oocytes developed to the next stage but only 1 oocyte (5%) in the group of 1.5 kV/cm pulse continued to develop to the blastocyst stage. In the next variant (fourth variant) only 5–10% of oocytes developed to the morula stage, and then they did not continue their development. In the “extreme” fifth variant, oocytes stopped to develop at the stage of 2–12 b (about 25%) and the percentage of degenerated oocytes increased dramatically (about 60%).

The analysis of all experimental variants indicates gradual and slight increases in the percentage of degenerating oocytes.

In this study, it was found that the electric field parameters from 1 pulse of 30 μ sec to 3 pulses of 30 μ sec had no visible effect on the parthenogenetic development of rabbit oocytes. We observed that the rabbit oocytes were adversely affected by the parameters of electric field that exceeded 3 pulses of 60 μ sec (fourth variant) when none of the oocytes continued development to the morula stage and 3 pulses of 60 μ sec and 3.5 kV/cm when all oocytes stopped their development at the 2 to 12 cell stage.

REFERENCES

- Cibelli J.B., Stice S.L., Golueke P.J., Kane J.J., Jerry J., Balckwell C., Abel Ponce de Leon F., Robl J.M. (1998): Cloned transgenic calves produced from nonquiescent foetal fibroblasts. *Science*, *280*, 1256–1258.
- Collas P., Robl J.M. (1990): Factors affecting the efficiency of nuclear transplantation in the rabbit embryo. *Biol. Reprod.*, *43*, 877–884.
- Collas P., Balise J.J., Hofman G.A., Robl J.M. (1989): Electrical activation of mouse oocytes. *Theriogenology*, *32*, 835–844.
- Dinneys A., Dai Y., Barber M., Liu L., Xu Jie, Zhou P., Yang X. (2001): Development of cloned embryos from adult rabbit fibroblasts: effect of activation treatment and donor cell preparation. *Biol. Reprod.*, *64*, 257–263.
- Escriba M.J., Garcia-Ximenez F. (1999): Electroactivation of rabbit oocytes in a hypotonic pulsing medium and parthenogenetic *in vitro* development without cytochalasin B-diploidizing pretreatment. *Theriogenology*, *51*, 963–973.
- Escriba M.J., Garcia-Ximenez F. (2000a): Influence of sequence duration and number of electrical pulses upon rabbit oocyte activation and parthenogenetic *in vitro* development. *Anim. Reprod. Sci.*, *59*, 99–107.
- Escriba M.J., Garcia-Ximenez F. (2000b): Use of variable electrical pulsing sequence in rabbit oocyte activation. *Reprod. Nutr. Dev.*, *40*, 261–169.
- Gruppen Ch.G., Verma P., Du Z.T., McIlfatric S.M., Ashman R.J., Nottle M.B. (1999): Activation of *in vivo*- and *in vitro*-derived porcine oocytes by using multiple electrical pulses. *Reprod. Fertil. Dev.*, *11*, 457–462.
- Hyun S., Lee B., Lee G., Lee E., Lim J., Kang S., Hwang W. (2003): Recruit of porcine oocytes excluded from nuclear transfer program for the production of embryos following parthenogenetic activation. *J. Vet. Med. Sci.*, *65*, 51–56.
- Liu L., Moor R.M. (1997): Factors affecting electrical activation of porcine oocyte matured *in vitro*. *Anim. Reprod. Sci.*, *48*, 67–80.
- Machaty Z., Prather R. (1998): Strategies for activating nuclear transfer oocytes. *Reprod. Fertil. Dev.*, *10*, 599–613.
- Modliński A., Smorąg Z. (1991): Preimplantation development of rabbit embryos after transfer of embryonic nuclei into different cytoplasmic environment. *Mol. Reprod. Dev.*, *28*, 361–372.
- Onodera M., Tsunoda Y. (1989): Parthenogenetic activation of mouse and rabbit eggs by electric stimulation *in vitro*. *Gamete Res.*, *22*, 277–283.

- Ozil J.P. (1990): The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. *Development*, 109, 117–127.
- Prather R.S., Barnes F.L., Sims M.M., Robl J.M., Eyestone W.H., First N.L. (1987): Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. *Biol. Reprod.*, 37, 859–866.
- Prochazka R., Kanka J., Sutovsky P., Fulka J., Motlik J. (1992): Development of pronuclei in pig oocytes activated by a single electric pulse. *J. Reprod. Fertil.*, 96, 725–734.
- Smith L.C., Wilmut I. (1989): Influence of nuclear and cytoplasmic activity on the development *in vivo* of sheep embryos after nuclear transplantation. *Biol. Reprod.*, 40, 1027–1035.
- Stice S.L., Robl J.M. (1988): Nuclear reprogramming in nuclear transplanted rabbit embryos. *Biol. Reprod.*, 39, 657–664.
- Tarkowski A. (1971): Recent studies on parthenogenesis in the mouse. *J. Reprod. Fertil., Suppl.*, 14, 31–39.
- Tarkowski A.K., Witkowska A., Nowicka J. (1970): Experimental parthenogenesis in the mouse. *Nature*, 226, 162–165.
- Wells D.N., Misica P.M., Tervit H.R. (1999): Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod.*, 60, 996–1005.
- Zimmermann U., Vienken J. (1982): Electric field-induced cell-to-cell fusion. *J. Membr. Biol.*, 67, 165–182.

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