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Effect of IP3 and ryanodine treatments on the development of bovine parthenogenetic and reconstructed embryos

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For parthenogenetic activation as a model system of nuclear transfer, microinjection and electroporation as activation treatments in bovine metaphase II oocytes were administered to each of three groups as follows: control group (treatments with Ca^{2+}, Mg^{2+}-free PBS+100 μM EGTA), IP3 group (control+25 μM IP3) and IP3+ ryanodine group (control+25 μM IP3+10 mM ryanodine). In experiments using microinjection, no significant differences were observed between any of the developmental stages of the electroporation experiment. For electroporation, cleavage rates were significantly higher in the IP3+ryanodine group than in the IP3 or control group (85.6% vs 73.7% or 67.6%, respectively). In the subsequent stages of embryonic development, such as morula and blastocyst formation, the IP3 and ryanodine group exhibited significantly higher rates of morula fomation than the IP3 or control groups (40.6% vs 24.2% or 16.7%, respectively). Similarly, the rate of blastocyst formation in the IP3+ryanodine group was significantly higher than the control group (16.3% vs 6.9%) but did not differ significantly from the IP3 group (16.3% vs 9.5%). In nuclear transfer, activation was performed at 30 hpm by microinjection and elecroporation with 25 μM IP3+ 10 mM ryanodine followed by 6-DMAP treatment. No significant differences were observed at any stage of embryonic development and none of the embryos activated by electroporation reached either the morula or blastocyst stage. However, 3.8% and 1.9% of embryos activated by microinjection sucessfully developed to the morula and blastocyst stages, respectively. In conclusion, activation treatments using IP3 and ryanodine are able to support the development of bovine parthenogenetic and reconstructed embryos.

Key words: Bovine, microinjection, electroporation, IP3, ryanodine, activation

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Introduction

In mammalian eggs, the mimicking of fertilization Ca^{2+} transients and oscillations has been widely applied as a means of achieving artificial activation of oocytes in nuclear transplantation experiments [4,22] and parthenogenesis using Ca^{2+} electroporation [28], ethanol [21], A23187 [25], sperm factor injection [27] and ionomycin [9]. The factors affecting the efficiency of nuclear transplantation are the enucleation of recipient oocytes, fusion, activation of the oocyte and reprogramming of the transferred nucleus. and activation has been suggested to be the factor responsible for the greatest loss of efficiency [5].

Fertilized mammalian eggs exhibit a series of multiple Ca^{2+} transients, as demonstrated in the hamster [7], mouse [10], pig [26] and cow. These Ca^{2+} oscillations persist for several hours, or until pronuclear formation [30]. These Ca^{2+} rises are required to induce egg activation, which consists of a sequence of events that includes cortical granule exocytosis, resumption of meiosis and the extrusion of the second polar body, pronuclear formaton, DNA synthesis and the first mitotic cleavage [10,24].

The origin of the Ca^{2+} increase is the release of Ca^{2+} from intracellular stores [8] and is generally attributed to the endoplasmic reticulum (ER). Repetitive Ca^{2+} transients occur as a result of the positive feedback mechanisms built into the oocyte`s calcium signaling system, which involves the modulated release and re-uptake of Ca^{2+} by the intracellular stores [33]. The increase in the concentration of intracellular free Ca^{2+} at the time of fertilization triggers the activation of the calmodulin-dependent protein kinase II (CaM KII). This in turn results in the inactivation of maturation promoting factor (MPF) and cytostatic factor (CSF) [18]. MAP kinase activity also decreases after oocyte activation, and high levels of MAP kinase activity have been found to be incompatible with pronuclear formation in fertilized mouse eggs, even after a decline in MPF activity [17].

Calcium release may occur via two-types of Ca^{2+} channels located on the surface of the ER : ryanodine and

IP3 receptors. Fertilization induces the intracellular release of calcium by activating these two kinds of calcium receptors [35]. The IP3 channels are gated by the phosphoinositide messenger IP3, whereas ryanodine receptors are opened by Ca^{2+} and cyclic-ADP ribose [33]. The plant alkaloid ryanodine has been demonstrated to bind to ryanodine receptor and to induce Ca^{2+} release [3]. Both of these pathways can produce regenerative Ca^{2+} oscillation [2,14,31]. At least three isoforms of both ryanodine and IP3 receptors have been identified and the existence of both receptors, and different isoforms, have been observed in both excitable and nonexitable cells [32,1,6]. Staining of ryanodine and IP3 receptors revealed that an extremely small number of both are present in GV-intact oocytes. As oocytes progress to MI, the intensity of receptor expression increased, but highest intensity was detected in MII matured bovine oocytes [35]. Ryanodine-generated Ca^{2+} release has been detected in sea urchin [13,23], mouse [29], bovine [34] and porcine oocytes [12].

Microinjection of IP3 was reported to evoke single or repetitive Ca^{2+} transients that induced various degrees of activation in a wide variety of species including mollusca, echinoderms, tunicates, fish, frogs, and mammals [16,19]. Micronjection of 250 nM of IP3 or 200 μM of ryanodine and 10 μM of inomycin treatment triggered similar intracellular calcium release. The rates of pronuclear formation and cleavage induced by 250 nM IP3 were 52% and 51% (IP3) and 60%, 54%(ryanodine) respectively [35].

Electrical stimulation is commonly used for oocyte activation and membrane fusion is used in the current nuclear transfer regimens in mammals. It has been postulated that short, high-voltage DC electric field pulses applied to eukaryotic cell plasma membranes cause the destabilization of the phospholipid bilayer, which results in the formation of temporary pores in the plasma membrane, thus allowing an exchange of extracellular and intracellular ions and macromolecules [36]. Extracellular Ca^{2+} electroporation (i.e. the electric pulse-induced formation of pores in the plasma membrane) has been demonstrated to induce oocyte activation in several species [20,5,22]. In addition to the influx of Ca^{2+} associated with electroporation, we cannot exclude the possibility that the increase in Ca^{2+} may be attributed to a release from intracellular stores [11]. In rabbit, electroporation of 25 mM IP3 in Ca^{2+} and Mg^{2+}-free PBS followed by 6-DMAP treatment, induced high rates of cleavage and blastocyst formation [15].

It has not yet been reported whether commonly used activation treatments, such as ionophore (ionomycin), ethanol and electric stimulation can induce IP3 and ryanodine receptor-mediated Ca^{2+} release. In this study, to stimulate IP3 and ryanodine receptors, microinjection and electroporation treatments with exogenous IP3 and ryanodine were used for oocyte activation.

Therefore, this study was conducted 1) to evaluate the efficiency of the parthenogenetic activation by IP3 and ryanodine microinjection or electroporation followed by 6-DMAP using metaphase II bovine oocytes, and 2) to determine whether IP3 and ryanodine microinjection or electroporation followed by 6-DMAP can lead to the development of bovine reconstructed embryos derived from nuclear transfer.

Materials and Methods

In vitro maturation

The bovine oocytes used in this study were obtained from bovine ovaries collected at a local slaughterhouse and transported at room temperature to the labaratory within 2 hour of slaughter. Oocytes were aspirated from 2 to 8 mm follicles and those with intact layers of cumlus cells and evenly shaded cytoplasm were selected and washed 3 times with Hepes-buffered tissue culture medium 199 (Hepes TCM 199; Gibco, Life technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM $NaHCO_3$ (Sigma, St. Louis, USA), 0.5% bovine serum albumin (BSA, Gibco) and 1% penicillin-streptomycin (Sigma). Approximately 40 COCs (cumulus-oocytes complexes) were subsequently placed in 4 well-dishes containing 450 μl of maturation medium which consists of TCM-199 supplemented with 10% FBS, 0.005 AU/ml FSH (Antrin, Teikoku, Japan), 1 μg/ml estradiol (Sigma), 1 mM sodium pyruvate (Sigma) and 1% penicillin-streptomycin per well, and cultured at 39°C in a humidified atmosphere of 5% CO_2 in air for 22 hours.

22 hours after the initiation of maturation, oocytes were completely stripped of their cumulus cells by gentle mouth-pipetting in Hepes-buffered CRaa-Washing medium supplemented with 0.1% hyaluronidase (Sigma) and 10% FBS. Oocytes with an extruded first polar body were selected for use in the experiment. For parthenogenetic activation, matured oocytes were placed in Hepes-buffered CRaa-Wash medium supplemented with 10% FBS for 8 hours at room temperature.

Enucleation of recipient oocytes

After a denuding process, cumulus-free oocytes were placed in a 4 μl drop of CRaa-Wash medium supplemented with 10% FBS on a micromanipulation chamber (Falcon). The zona pellucida adjacent to the first polar body was slit with a fine glass needle and the oocytes were squeezed to remove the first polar body and approximately 10% of the cytoplasm with a metaphase II plate. Enucleation was confirmed by visualizing the karyoplast stained with Hoechst 33342 (Sigma) under ultraviolet light at a 100X magnification. The enucleated oocytes were placed in TCM-199 supplemented with 10% FBS, 1 mM sodium

pyruvate (Sigma) and 1% penicillin-streptomycin for up to 1hour until injection of the donor cells.

Preparation of donor cells for nuclear transfer

Cell lines were obtained from the skin of an adult cow. The excised ear skin tissues were washed with Dulbecco's phosphate buffered saline (DPBS, Gibco) and finely cut into numerous small pieces. These tissues were enzymatically digested with 0.25% trypsin-EDTA (Gibco) in phosphate buffered saline for about 1 hour at 38°C in a humidified atmosphere of 5% CO_2. Digested tissues were washed in PBS by repeated centrifugation and Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS was added to the pellet. The cell suspension was placed in culture dishes in a humidified atmosphere of 5% CO_2 for approximately 4 days until the monolayer had formed. To maintain the cell lines, they were trypsinized for 30 sec and passaged into new dishes to synchronoze the cell cycle at the G0 stage and cultured in a 0.5% serum containing media. One day after routine passage, the culture medium was replaced with fresh culture medium containing only 0.5% FBS. Cells were subsequently cultured for further 2-21 days before being used for nuclear transfer. Immediately before injection, a single cell suspension of the donor cells was prepared by standard trypsinization. The cell were pelleted and resuspended in PBS with 0.5% FBS and maintained in this medium until the donor cells were injected.

Injection of donor cells into recipient oocytes

After culturing the enucleated oocytes for 1 hour in TCM-199 medium, the oocytes were washed several times in CRaa-Wash medium containg 10% FBS and 100 μg/ml phytohemagglutinin (Sigma), which supports firm attachment between the donor cells and recipient oocytes. As the recipient oocytes were placed in a 4 μl drop of CRaa-W medium containg 10% FBS and 100 μg/ml phytohemagglutinin (Sigma), donor cells were placed in other 4 μl drop of phosphate buffered saline (PBS; Gibco BRL, Life Technologies, NY, USA) supplemented with 0.5% FBS. Each donor cell was injected into the space between the zona pellucida and the cytoplast membrane through a slit that had been made previously during the enucleation process using a 30 um (approximate external diameter) pipette.

Cell fusion for nuclear transfer

Injected donor cells and recipient oocytes were electrically fused at 24 h post maturation in a buffer solution containing 0.28 M mannitol (Sigma), 0.5 mM HEPES, 0.05% fatty acid-free BSA and 0.1 mM magnesium in a chamber with two stainless steel electrodes 3.4 mm apart. The reconstructed embryos were gently placed between the two electrodes and the surface of the contact surface between the donor cell and recipient oocyte was manually aligned so that it was parallel with electrodes. Electrical pulses were then applied with a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA), and monitored with a BTX Optimizer-Graphic Pulse Anlayzer. Cell fusion was induced with two DC pulses of 1.75 kv/cm of 15usec duration and 1 sec apart. After fusion, these embryos were placed in CRaa-W medium supplemented with 10% FBS for 6 hours at room temperature, after which only fused embryos were selected for the activation process.

Microinjection of IP3 and ryanodine for activation

For the parthenogenetic activation of bovine oocytes, metaphase II oocytes aged for 8 hours were placed in 4 μl drop of CRaa-Wash medium supplemented with 10% FBS. Microinjection was performed into the cytoplasm using 25 μM IP3 (extracellular concentration) (Molecular probes, Oregon, USA) alone or 25 μM IP3 and 10 mM ryanodine (extracellular concentration)(Calbiochem, CA, USA) dissolved in Ca^{2+}, Mg^{2+}-free PBS supplemented with 100 μM EGTA (Sigma) using 10 μm (external diameter) in vitro fertilization pipette (Humagen, Virginia, USA) connected to a Narishige microinjector. The control group was microinjected with Ca^{2+}, Mg^{2+} -free PBS supplemented with 100 μM EGTA. Oocyte volume was standarized at 800-900 pl and the injection volume used was approximately 8-9 pl, which is about 1% of the oocyte volume. Reconstructed embryos that were placed in CRaa-Wash medium supplemented with 10% FBS for 6 hours after fusion were microinjected with 25 μM of IP3 and 10 mM of ryanodine together, as described in parthenogenetic activation. All oocytes in each of the experimental groups were incubated in CRaa D I supplemented with 1.9 mM DMAP for 4 hours at 39°C in a humidified atmosphere of 5% CO_2 and air.

Electroporation of IP3 and ryanodine for activation

For the parthenogenetic activation of bovine oocytes, 30 hpm metaphase II oocytes were washed in Ca^{2+} and Mg^{2+} free PBS several times and transferred to a electroporation chamber with two stainless steel electrodes with 3.4 mm apart. Electroporation was performed in a buffer solution containing 25 μM of IP3 alone, or 25 μM of IP3 and 10 mM of ryanodine dissolved in Ca^{2+} and Mg^{2+}-free PBS supplemented with 100 μM EGTA in an electroporation chamber, with two DC pulses of 1.75 kV/cm for 15 usec duration, 1 sec apart. Electrical pulses were applied with a BTX Electro Cell Manipulator 2001, and monitored with a BTX Optimizer-Graphic Pulse Anlayzer. Electroporation of the control group was performed in Ca^{2+} and Mg^{2+} -free PBS supplemented with 100 μM of EGTA.

Reconstructed embryos that were placed in the CRaa-Wash medium, supplemented with 10% FBS for 6 hours

after fusion, were subsequently electroporated with 25 μM of IP3 and 10 mM ryanodine, as described for parthenogenetic activation. All oocytes in each of the experimental groups were incubated in CRaa DI supplemented with 1.9 mM DMAP for 4 hours at 39°C in a humidified atmosphere of 5% CO_2 and air.

***In Vitro* culture**

Parthenogenetically activated oocytes and reconstructed embryos after 6-DMAP treatment were cultured in specifically modified CRaa medium for this experiment in a humidified atmosphere of 5% CO_2, 7% O_2 and air. For the first three days of culture, approximately 10 embryos were grouped together and placed in a 25 ul drop of CRaa D I. Embryos were then moved to CRaa D II on the fourth day of culture for final development. Cleavage rates were examined at 48 h after culture and each developmental stage from 2 cell to blastocyst was monitored every day.

Statistical analysis

Multiple comparisons (LSD) were performed using Generalized Linear Models in the SAS 6.12 program ($P<0.05$).

Results

Experiment 1. Development of parthenogenetically activated oocytes by microinjection with IP3 alone, or IP3 and ryanodine together, followed by 6-DMAP treatment.

As shown in table 1, the rate of cleavage in the control group was not significantly different from that of other groups, but tended to be slightly higher than that the cleavage rates observed in the IP3 and IP3 + ryanodine groups espectively(69.8% vs 61.1%, 66.7%). A similar result was observed in the rate of development to the 4 cell stage. The rate of later embryonic developments from 8 cell to blastocyst were also not siginificantly different in the 3 groups, despite IP3 + ryanodine and IP3 groups showed a higher rate of morula and blastocyst formation than control group (morula:16.7%, 16.0% vs 14.0%, blastocyst:8.8%, 6.9% and 5.8%, respectively).

Experiment 2. Development of Parthenogenetically activated oocytes by electroporation with IP3 alone or IP3 and ryanodine together followed by 6-DMAP treatment.

As described in table 2, the cleavage rate of the IP3 + ryanodine group was significantly higher than that observed for the IP3 and control groups (85.6% vs 73.7%, 67.6%, respectively). The rate of development to the 4 cell and 8 cell stage embryos, was similar to the result obtained for cleavage rate. During the later stages of embryonic development, such as morula and blastocyst formation, the IP3 + ryanodine group exhibited a significantly higher rate of morula fomation than was observed in the IP3 and control group(40.6% vs 24.2%, 16.7%, respectively). Furthermore, the rate of blastocyst formation in the IP3 + ryanodine group was significantly higher than that of the control group (16.3% vs 6.9%) but did not significantly differ from IP3 group (16.3% vs 9.5%).

Table 1. developmental rate of parthenogenetic embryos activated by Microinjection

Activation protocols	No. of oocytes	cleavage(%)	4 cell(%)	8 cell(%)	Mo*(%)	BL**(%)
Control	86	60(69.8)	47(54.7)	22(25.6)	12(14.0)	5(5.8)
IP3	144	88(61.1)	64(44.4)	41(28.5)	23(16.0)	10(6.9)
IP3+Ryanodine	102	68(66.7)	51(50.0)	30(29.4)	17(16.7)	9(8.8)

Model effect of the treatments on the number of cleavage, 4 cell, 8 cell, Mo and BL, which was indicated as a P value, was 0.3810, 0.3139, 0.8340, 0.8711 and 0.8391, respectively.
*Morula
**Blastocyst

Table 2. Parthenogenetic development of oocytes activated by electroporation

Activation protocols	No. of oocytes	cleavage(%)	4 cell(%)	8 cell(%)	Mo*(%)	BL**(%)
Control	102	69(67.6)^{a}	52(51.0)^{a}	31(30.4)^{a}	17(16.7)^{a}	7(6.9)^{a}
IP3	95	70(73.7)^{a}	54(56.8)^{a}	33(34.7)^{a}	23(24.2)^{a}	9(9.5)^{ab}
IP3+Ryanodine	160	137(85.6)^{b}	118(73.8)^{b}	80(50.0)^{b}	65(40.6)^{b}	26(16.3)^{b}

$^{a-b}$Within a column, values with different superscripts were significantly different($p<0.05$, LSD)
* Morula
**Blastocyst

Table 3. Development of reconstructed embryos after activation.

Activation protocols	No. of oocytes	cleavage(%)	4 cell(%)	8 cell(%)	Mo*(%)	BL**(%)
IP3+Ryanodine microinjection	53	28(52.8)	13(24.5)	5(9.4)	2(3.8)	1(1.9)
IP3+Ryanodine electroporation	56	33(58.9)	19(33.9)	9(16.1)	0(0)	0(0)

Model effects of the treatments on the number of cleavage, 4 cell, 8 cell, Mo and BL, which was indicated as a P value, was 0.5259, 0.2857, 0.3050, 0.1450 and 0.3062, respectively.
*Morula
**Blastocyst

Experiment 3. Development of reconstructed embryos activated by IP3 and ryanodine microinjection or electroporation

To ascertain whether IP3 and ryanodine treatments influence the activation of reconstructed embryos, embryos were activated by IP3 and ryanodine microinjection or electroporation, which showed the best results in terms of morula and blastocyst. No significant difference was observed in the early embryonic development, particularly in cleavage rates between microinjection and electroporation group (52.8% vs 58.9%, respectively). None of the embryos activated by electroporation matured to form morula and blastocysts. However, 3.8% and 1.9% of the embryos activated by microinjection sucessfully developed to the morula and blastocyst stages.

Discussion

At fertilization, spermatozoa not only deliver DNA to the oocyte to restore diploidy, but they also trigger a series of intracellular processes essential to embryogenesis. In several mammalian species, sperm penetration produces transient, but periodic Ca^{2+} increases that may last for several hours. In bovine oocytes sperm penetration causes the generation of multiple transient increases in intracellular calcium.

Two kinds of receptor located on the surface of ER (IP3 receptor, ryanodine receptor) have been clearly identified in bovine oocytes. Given that these receptors are thought to play a key role in oocyte activation, exogenous IP3 and ryanodine were selected and assessed to determine whether they could promote full oocyte activation in bovine parthenogenetic and reconstructed embryos. Microinjection of 50-250 μM of IP3 into M II bovine oocytes has been demonstrated to cause either single, or repetitive, intracellular calcium rises and 100-200 mM of ryanodine microinjection has also been demonstrated to cause increases in Ca^{2+} levels with peak values of calcium release similar to treatment with 10 μM ionomycin, which is a potent Ca^{2+} ionophore and usually selected for mobilization of intracellular Ca^{2+} [35]. In addition, electroporation of rabbit oocytes in Ca^{2+}, Mg^{2+} free PBS supplemented with 25 μM IP3 and 100 mM EGTA with 1.4 kV/cm, two 15 usec DC pulses spaced 1 sec apart followed by 6-DMAP treaement, resulted in higher rate of cleavage and blastocyst fomation than ionomycin treatment followed by 6-DMAP treatment and successfully supported the development of reconstructed rabbit embryos to the blastocyst stage [15].

Given that there are relatively few reports pertaining to IP3 and ryanodine microinjection or electroporation followed by 6-DMAP treatment for the development of bovine parthenogenetic and reconstructed embryos, this study was undertaken to investigate the efficiency of an activation protocol using IP3 and ryanodine. In the first experiment, the development of parthenogenetically activated oocytes by microinjection of 25 μM IP3 alone, or 25 μM IP3 and 10 mM ryanodine together, followed by 6-DMAP treatment was assessed. Before activation treatment, denuded oocytes were aged for 8 hours at room temperature. The omission of this aging period resulted in significantly decreased cleavage rates with none of the embryos reaching the blastocyst stage. It is thought that aging of oocytes at room temperature is crucial for successful microinjection. The cleavage rate as well as the percentage of oocytes that developed to the 4 cell stage was higher than other two treatment groups. This could be due to possible mechanical damage to metaphase II plate of the oocytes during handling the injection pipette, which would have disrupted cleavage. On the other hand, the rate of development from 8 cell stage to blastocyst formation increased slightly after the injection of IP3. Similarly, the addition of ryanodine to the injection medium elevated the rate at which the later stages developed when compared to the rate of development with IP3 alone. These results indicate that the administration of IP3 and ryanodine by microinjection, may play a role in the mobilization of Ca^{2+} stores, and affect the developmental competence. This hypothesis was effectively borne out by experiment 2.

Development of parthenogenetically activated oocytes by electroporation of 25 μM IP3 alone or 25 μM IP3 and 10 mM ryanodine together followed by 6-DMAP treatment, was examined in experiment 2. The oocytes

electroporated in Ca^{2+}, Mg^{2+}-free PBS supplemented with 25 μM IP3 and 100 μM EGTA were proven to elicit somewhat higher rates than were observed in the developmental stages of the control group. Furthermore, the addition of ryanodine made a siginificant difference with other two groups from cleavage to morula stage. Although there is a possibility of Ca^{2+} release from ER only by electrical stimulus, we postulate that IP3 and ryanodine were transported into the cytoplasm via temporary pores in the plasma membrane and these compounds rapidly diffuse into the cytoplasm, where they bind to specific receptors thereby mobilizing Ca^{2+} from intracellular stores. In experiment 2, the efficiency of activation was not affected by the oocyte age. One problem is that a relatively small proportion of the oocytes acitivated by electroporation were lysed (data not shown). This means that the conditions surrounding the application of an electrical stimulus as a means of achieving activation were not entirely appropriate in so far as the conservation of intact oocytes was concerned. Therefore, electroporation with IP3 and ryanodine can be applied to bovine parthenogenetic activation.

These two activation protocols were applied to reconstructed embryos in experiment 3. As shown in table 1 and 2, activation methods that give rise to higher efficiencies, particularly in the later stages of development when compared to the previous two experimental procedures, were IP3 and ryanodine microinjection and electroporation. Therefore, these two methods were selected and applied to NT. Oocytes were aged for 6 hours at room temperature, after fusion, in order to improve the activation efficiency of both groups. Although cytochalasin B, a microfilament polymerization inhibotor, is commonly used to aid enucleation, it was excluded in this experiment. Based on the findings of this work, when oocytes were enucleated in the medium supplemented with cytochalasin B, cleavage rates were low and further development to the later embryonic stages hardly occured. There were no significant differences between the two groups in all stages of development in experiment 3, and when these data were compared with parthenogenetic activation, lowered activation efficiencies were observed with only 1.9% of embryos activated by IP3 and ryanodine microinjection reached blastocyst stage while none of embryos activated by IP3 ryanodine elecroporation becoming morula and blastocyst. This contrasted with the electroporation experimental procedure in which none of the embryos activated by electroporation reached either morula or blastocyst stages. Although we were unable to resolve this problem, It may be postulated that the removal of approxiamately 10% of the oocyte cytoplasm may have reduced IP3 and ryanodine receptor, thus decreasing the activation efficiency. In addition, the expression pattern of IP3 and ryanodine receptors depends on the stage of meiosis and the depletion of these receptors associated with the removal of metaphase spindle, can not be excluded. Further study is required to investigate the modulation of these receptors after enucleation. In this study we suggest new activation protocols using IP3 and ryanodine, but the problem of low efficiency in nuclear transfer should be addressed through further study.

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