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Parthenogenetic and Nuclear Transfer Rabbit Embryo Development and Apoptosis After Activation Treatments

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ABSTRACT Previous studies mainly evaluated the effect of culture conditions on preimplantation embryo apoptosis. In order to inhibit apoptosis of nuclear transfer (NT) embryos, putative apoptosis inhibitors were used to treat donor cells. However, little is known about the effect of activation treatments on embryo apoptosis. We firstly investigated the effect of various parthenogenetic activation (PA) treatments on embryo development, blastocyst cell number, and apoptosis, and then one of these activation treatments proved to be most efficient was selected for activation rabbit NT embryos. The activation by electrical pulses and 30 min later, electroporation with 25 μM D-myoinositol 1,4,5-trisphosphate (IP3) in Ca^{2+}- and Mg^{2+}-free PBS, then exposure to 2.0 mM 6-dimethylaminopurine (6-DMAP) for 3 hr effectively activated rabbit oocytes, and resulted in significantly a higher blastocyst development rate (72.7%) and total cell number (175 ± 14.1), and markedly lower apoptosis level of blastocyst (4.3 ± 0.5) than all the other groups. When the same activation protocol was applied in NT embryo activation, we found that exposure of the embryos to 6-DMAP for 3 hr could decrease the apoptosis level of blastocyst and increase blastocyst rate and cell number. The results demonstrate that oocyte activation affects not only embryo development and quality but also embryo apoptosis. *Mol. Reprod. Dev. 72: 48–53, 2005.* © 2005 Wiley-Liss, Inc.

Key Words: nuclear transfer; embryo development; embryo apoptosis; activation

INTRODUCTION

The production of genetically identical mammals by somatic cell nuclear transfer (NT) is now a reality (Campbell et al., 1996; Wilmut et al., 1997; Cibelli et al., 1998; Kato et al., 1998; Wakayama et al., 1998). However, low efficiencies and high embryo, fetal, and neonatal losses necessitate further research into the basic mechanisms controlling the early preimplantation development of NT embryos. Previous research has suggested that apoptosis plays a potential role in early embryo loss (Devreker and Hardy, 1997; Wuu et al., 1999; Matwee et al., 2001). Apoptotic level and cell number of blastocysts are important parameters for evaluating embryo development and health (Brison and Schultz, 1997, 1998; De la Fuente and King, 1997). It is well documented that total cell number can be regarded as a valuable indicator of cattle embryo viability (Knijin et al., 2003). The function of apoptosis is to eliminate the minority of cells with abnormal, detrimental, or superfluous potential during the embryo cleavage stage (Pampfer and Donnay, 1999). Increased apoptosis is probably related to embryo losses and to lower developmental competence of in vitro fertilized and cultured embryos (Betts and King, 2001).

Although there have been many attempts to clone rabbit (Stice and Robl, 1988; Collas and Robl, 1990; Yang et al., 1992), so far only one group has successfully produced live offspring from rabbit cumulus cell nuclear transfer (Chesne et al., 2002). Since activation of the recipient oocyte is a crucial step in successful nuclear transfer techniques (Collas et al., 1993), incomplete activation of oocytes may in part be responsible for higher apoptosis in rabbit NT blastocysts than that in fertilized blastocysts (our unpublished data), and it may result in the difficulty of rabbit cloning. An optimal activation protocol may enhance better or complete reprogramming of the reconstructed embryo, which might further increase the chance of success in rabbit cloning.

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 (Collas and Robl, 1990) and parthenogenesis experiments using electroporation (Stice and Robl, 1988), ethanol (Presicce and Yang, 1994), A23187 (Balakier and Casper, 1993), ionomycin (Jones et al., 1995), and IP3 (Mitalipov et al., 1999).

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Apoptosis may be associated with activation of the embryo genome and may contribute to the blastomere fragmentation commonly observed in rabbit NT embryos. In order to determine the relationship between oocyte activation and blastocyst apoptosis, we investigated: (1) embryo development, blastocyst cell number, and apoptosis after activation treatments of the rabbit MII oocytes; and (2) development, cell number and apoptosis of NT embryos after activation by electrical pulses plus IP3 and 6-DMAP.

MATERIALS AND METHODS

Animals

Animal care and handling were in accordance with the policy on the Care and Use of Animals of the Ethical Committee, Institute of Zoology, Chinese Academy of Sciences. Female Japan Big Eared white rabbits (purchased from Laboratory Animal Center, Institute of Zoology, Chinese Academy of Sciences) were housed in stainless steel cages, and were fed with regular rabbit fodder and water ad libitum.

Oocyte Collection and Donor Cell Preparation

Mature female rabbits were superovulated by administering PMSG and hCG (Institute of Zoology, Chinese Academy of Sciences). Each rabbit was injected with 150 IU PMSG and 100 IU hCG 4 days after the PMSG injection. The oviducts were flushed with M2 medium (Sigma Chemical Company, St. Louis, MO) 14–18 hr after administration of hCG. Cumulus masses were treated shortly thereafter with 300 IU/ml of hyaluronidase (Sigma) in M2 medium. Cumulus cells were stripped from the oocytes by repeated gentle pipetting. The cumulus cells were washed three times in M199 medium and used as donor cells. Oocytes were then subjected to parthenogenetic activation or nuclear transfer experiments as discussed later.

Oocyte Activation and Culture

Cumulus-free MII oocytes (16–18 hr after hCG) were activated by double DC pulses of 1.2 kV/cm for 20 μsec spaced 1 sec apart in fusion medium and 30 min later, oocytes were randomly assigned to one of the two activation treatments: (1) electroporation (two 20-μsec DC pulses spaced 1 sec apart, 1.2 kV/cm) with IP3 (25 μM D-myoinositol 1,4,5-trisphosphate) in Ca^{2+}- and Mg^{2+}-free PBS plus 100 mM EGTA), then exposure to 2.0 mM 6-DMAP for 3 hr (EID); (2) culture in 2.0 mM 6-DMAP for 3 hr (ED). In the other three activation treatment groups, cumulus-free MII oocytes were randomly assigned to: (1) electroporation (two 20-μsec DC pulses spaced 1 sec apart, 1.2 kV/cm) with IP3 (25 μM D-myoinositol 1,4,5-trisphosphate) in Ca^{2+}- and Mg^{2+}-free PBS plus 100 mM EGTA), then exposure to 2.0 mM 6-DMAP for 3 hr (ID); (2) electroporation by double DC pulses of 1.2 kV/cm for 20 μs spaced 1 sec apart in fusion medium alone (EP, control); (3) electroporation (two 20-μsec DC pulses spaced 1 sec apart, 1.2 kV/cm) with IP3 (25 μM D-myoinositol 1,4,5-trisphosphate) in Ca^{2+}- and Mg^{2+}-free PBS plus 100 mM EGTA) alone (IP3, control). In these experiments electroporation medium and field strength were the same as those used for nuclear transfer. Embryos were cultured for 4.0 days in TCM 199 and 10% FBS.

Preparation of Recipient Cytoplasm

Procedure of recipient oocyte preparation has been described previously (Yang et al., 2003). The cumulus-free eggs (14 hr post hCG) were transferred to M2 medium containing 7.5 μg/ml cytochalasin B (Sigma Chemical Co.), 7.5 μg/ml Hoechst 33342 (Sigma Chemical Co.) and 10% FBS for 10 min and used for micromanipulation. For enucleation, a small amount of cytoplasm from the area beneath the first polar body containing the meiotic spindle was aspirated using a 20–25 μm glass pipette, and then the aspirated karyoplast was exposed to ultraviolet light to confirm the presence of nucleus. Only the oocytes from which the chromosomes were removed were used for nuclear transfer (Chen et al., 1999).

Nuclear Transfer, Activation, and Embryo Culture

Rabbit cumulus derived from cumulus–oocyte complexes were used as nuclear donors. The nuclear transfer (NT) was conducted as previously described (Chen et al., 1999, 2002). A single donor cell was placed in the perivitelline space. The couplets were transferred to a fusion chamber consisting of two wires, 1 mm apart and overlaid with the 100 μl fusion of medium (0.25M sorbitol, 0.5 mM magnesium acetate, 0.1 mM calcium acetate, 0.5 mM HEPES, and 100 mg/100 ml BSA). Fusion was induced by double DC pulses of 1.4 kV/cm for 80 μs with an ECM2001 Electrocell Manipulator (BTX, Inc., San Diego, CA). Couplets were then washed in M199 (Gibco BRL, NY) supplemented with 10% FBS for three times, and incubated in the same medium for 30 min at 38°C in a humidified air containing 5% CO_2. Couplets were checked for fusion under an inverted microscope, and fused couplets activation by double DC pulses of 1.2 kV/cm for 20 μs spaced 1 sec apart in fusion medium and 30 min later, NT embryos were activated by electroporation (two 20-μsec DC pulses spaced 1 sec apart, 1.2 kV/cm) with IP3 (25 μM D-myoinositol 1,4,5-trisphosphate) in Ca^{2+}- and Mg^{2+}-free PBS plus 100 mM EGTA), then randomly assigned to be cultured in 6-DMAP for 4, 3, or 2 hr. After these various treatments, the cloned embryos were cultured for 4 days in M199 (Gibco BRL) supplemented with 10% FBS.

TUNEL and Confocal Microscopy

Day 4 blastocysts were assessed and scored by stereomicroscopy. The blastocysts were randomly selected and fixed. The PA and NT blastocysts were washed three times in PBS/PVP (PBS supplemented with 0.1% polyvinyl-pyrrolidone) and fixed in 4% (v/v) paraformaldehyde solution for 2 hr at room temperature. Membrane was permeated in 0.1% Triton X-100 in 0.1% citrate solution for 1 hr at room temperature. A terminal

deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL) assay was used to assess the presence of apoptotic cells (in situ Cell Death Detection Kit, TMR red; Roche; Mannheim, Germany). Fixed blastocysts were incubated in TUNEL reaction medium for 1 hr at 38.5°C in the dark. The broken DNA ends of the embryo cells were labeled with TdT and fluorescein-dUTP. After the reaction was stopped, labeled embryos were all incubated in 0.1 mg/ ml of RNase A (Sigma), and DNA was counterstained with 10 mg/ml propidium iodide (PI; Sigma) for 5 min. As positive controls for TUNEL, fixed embryos were incubated in RQ1 Rnase-Free DNase (Promega, Madison, WI) for 40 min in the dark before TUNEL at the room temperature. The negative controls were generated by omitting terminal transferase in the reaction. In order to conserve spherical morphology, blastocysts were processed through an increasing gradient of anti-fade (Vector Laboratories, Burlingame, CA) and mounted on a glass slide. Slides were stored at −20°C up to 7 days before fluorescence microscopic evaluation. Scoring of nuclei in embryos was performed according to the criteria described below. Blastocysts were subsequently subjected to confocal laser-scanning microscopy on a Leica TCS4D microscope (Leica Laser Technik, GmbH, Heidelberg, Germany) using an argon/krypton laser at 488 and 568 nm and two-channel scanning for detection of fluorescein isothiocyanate and PI, respectively. Complete Z series of 20–25 optical sections at 3–4 mm intervals were acquired from each embryo using Leica Scanware software. With this sectioning interval, all nuclei appeared on at least two consecutive images, thereby assuring that all nuclei of an embryo were registered. Image stacks were reconstructed with a Silicon Graphics octane computer (SGI, Mountain View, CA) equipped with an Imaris image analysis software package (Bitplane, Zurich, Switzerland), and reconstructed confocal images were used for scoring of nuclei in each blastocyst.

Quantitative Analysis of TUNEL Labeling and Apoptosis

Digitally recombined, composite images were analyzed using the Imaris software. All 20–25 optical sections were divided using a standard grid over each layer to count all nuclei as a measure of the cell number of the embryo. Nuclei were scored for TUNEL labeling, signs of fragmentation, and condensation. Cells were judged to be apoptotic when the nucleus displayed both the biochemical feature (TUNEL labeling) and the morphological feature (Gjørret et al., 2003). The apoptotic index of the embryos was calculated as the percentage of apoptotic cells relative to the total number of cells.

Statistical Analysis

Data were analyzed using SPSS 11.0 statistical software. Random distribution of blastocysts was made in each experimental group and experiments were replicated at least ten times. Interaction analysis among experimental parameters was first performed. As no interaction was found, the data were subjected to analysis of variance (ANOVA) and protected least significant different (LSD) test using general linear models to determine differences among experimental groups. Data were analyzed using the Chi-square test (χ^2) for the developmental potential of activated oocytes and NT embryos. Statistical significance was determined when *P* value was less than 0.05.

RESULTS

Effect of various activation treatments on the subsequent development and apoptosis of rabbit MII oocytes were examined (Table 1). No blastocyst was produced from IP3 alone treatment. Activation by ED and ID resulted in significantly higher blastocyst development rate and cell number, and markedly lower apoptotic index than those in EP group. There were no significant differences in cleavage rate among ED, ID, and EP groups, but the cleavage rate in the three groups was markedly higher than that in IP3 alone group. Blastocyst development rate (72.7%) and cell number (175 ± 14.1) in EID group were strikingly higher and blastocyst apoptotic index (4.3 ± 0.5) was significantly lower than in all the other groups.

The cleavage rate, blastocyst development rate, cell number, and apoptosis of cloned embryos produced by different activation treatments were compared (Table 2). There was no significant difference in cleavage rate among the three groups, but the blastocyst development rate was greatly higher in EID (3 hr) group (55.9%) than that in EID (2 hr) group (21.7%) or EID

TABLE 1. Effect of Various Activation Treatments on the Embryo Development, Blastocyst Cell Number, and Apoptosis in Parthenogenetic (PA) Embryos

Group	No. of oocytes	No. (%) cleaved	No. (%) blastocysts	Cell number (n)	Apoptotic index % (n)
EID	88	79 (89.7)^{a}	64 (72.7)^{a}	175 ± 14.1^{a} (30)	4.3 ± 0.5^{a} (30)
ID	82	49 (59.8)^{a,b}	34 (41.5)^{b}	159 ± 18.1^{b} (23)	6.1 ± 3.1^{b} (23)
ED	105	60 (57.1)^{b}	39 (37.1)^{b}	161 ± 20.9^{b} (30)	7.2 ± 2.5^{b} (30)
EP	146	58 (39.7)^{b}	24 (16.4)^{c}	148 ± 13.5^{c} (24)	10.1 ± 1.6^{c} (24)
IP3 alone	107	29 (27.1)^{c}	0^{c}		

Cell number and apoptotic index are shown as mean ± SEM; n, number of blastocysts. Apoptotic index is calculated as the percentage of cells displaying both TUNEL labeling and fragmentations. EP, electrical pulses; ED, EP plus 6-DMAP; ID, inositol 1,4,5-triphosphate (IP3) plus 6-DMAP; EID, EP plus IP3 and 6-DMAP. Values with different superscripts within each column are significantly different (at least $P < 0.05$).

TABLE 2. Development, Cell Number, and Apoptosis of NT Embryos After Activation by EID

Group	No. fused	No. (%) cleaved	No. (%) blastocysts	Cell number (n)	Apoptotic index % (n)
EID (4 hr)	83	51 (61.4)[a]	27 (32.5)[a]	153 ± 15.6^{a} (30)	8.8 ± 3.0^{a} (30)
EID (3 hr)	111	67 (60.4)[a]	62 (55.9)[b]	174 ± 11.9^{b} (32)	5.3 ± 1.3^{b} (32)
EID (2 hr)	106	47 (44.3)[a]	23 (21.7)[a]	141 ± 21.9^{a} (23)	$7.2 \pm 2.1^{a,b}$ (23)

Cell number and apoptotic index are shown as mean ± SEM; n, number of blastocysts. Apoptotic index is calculated as the percentage of cells displaying both TUNEL labeling and fragmentations. EID, electrical pulses plus IP3 and 6-DMAP. Values with different superscripts within each column are significantly different (at least $P < 0.05$).

(4 hr) group (32.5%). Blastocyst total cell number (174 ± 11.9) of blastocysts in EID (3 hr) group was greatly higher than that in the other two groups and apoptotic index in this group (5.3 ± 1.3) was significantly lower than that in EID (4 hr) group (8.8 ± 3.0). There was no marked difference in apoptotic index between EID (3 hr) and EID (2 hr) groups.

Representative images of labeled PA and NT blastocysts for total cell counts and measurement of apoptosis were displayed in Figure 1.

DISCUSSION

The regulation of apoptosis in the preimplantation embryo is likely to be of critical importance for both embryo viability and later development (Brison, 2000). Most of previous studies about embryo apoptosis evaluated the effect of culture conditions on fertilized, parthengenetic or nuclear transfer embryos (Brison and Schultz, 1997; Herrler et al., 1998; Kawamura et al., 2003; Fabian et al., 2004; Park et al., 2004b). In order to inhibit apoptosis in NT embryos, treatment of donor somatic cells with putative apoptosis inhibitors was reported (Park et al., 2004a). However, there is little information about the effect of activation treatments on the preimplantation embryo apoptosis.

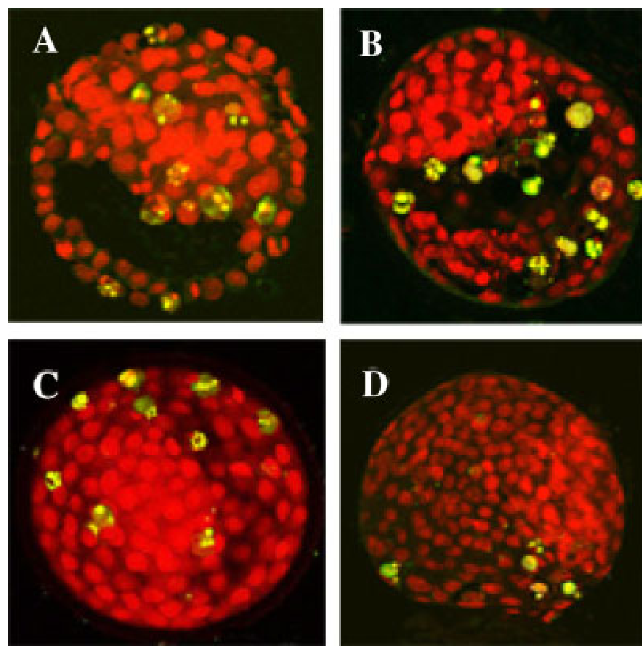

Fig. 1. Total cell number and apoptosis in rabbit blastocysts. The nucleus was stained by PI (red), fragmented nucleus was labeled by TUNEL reaction (green), and colocalization with PI was indicated as yellow. **A**: PA blastocyst consisting of 120 total cells and 10 apoptotic cells produced from electric pulses treatment (EP); (**B**) NT blastocyst consisting of 148 total cells and 12 apoptotic cells produced from the EP + IP3 + DMAP (4 hr) treatment; (**C**) PA blastocyst consisting 168 cells and 9 apoptotic cells produced from the EP + IP3 + DMAP treatment; (**D**) NT blastocyst consisting of 182 cells and 5 apoptotic cells produced from the EID (3 hr) treatment. Magnification was ×400 for all panels. [See color version online at www.interscience.wiley.com.]

Activation of recipient oocyte is a critical step for further development of NT embryo. In the present study, we used electrical pulses (EP), EP plus 6-DMAP (ED), inositol 1,4,5-triphosphate (IP3) plus 6-DMAP (ID), or EP plus IP3 and 6-DMAP (EID) to induce oocyte activation (Table 1). Results showed that the rate and cell number of blastocysts in EP group were greatly lower than those in ED group. This observation is consistent with a previous report that electrical stimulation alone could induce pronuclear formation, however, further development was compromised when compared to that resulted from combining electrical and chemical stimulation (Liu et al., 2004). The apoptotic index in EP group was greatly higher than that in ED group (Table 1). Liu et al. (2002) demonstrated that parthenogenetic activation and parthenogenesis themselves do not cause apoptosis, but haploidy formation increases the incidence of apoptosis in preimplantation embryos. The increased incidence of apoptosis observed in haploid parthenotes is not due to the lack of a paternal genome because diploid parthenotes, without a paternal genome, exhibit a low frequency of apoptosis, comparable to that of in vitro fertilized embryos (Liu et al., 2002). Sequential treatment with electroporation and 6-DMAP resulted in one diploid pronucleus without second polar body extrusion (Liu and Yang, 1999), produced diploid parthenotes. Thus, higher diploid parthenotes in ED group may be one reason resulting in lower apoptotic level in this group than that in EP group.

During oocyte activation, inactivation of maturation promoting factor (MPF) is a prerequisite to the resumption and completion of meiosis, subsequent pronuclear formation, and DNA synthesis (Collas et al., 1993; Verlhac et al., 1994; Moos et al., 1996). MPF activity, at least in young bovine and rabbit oocytes, is quickly restored with recondensation of chromosomes and reentry of activated oocytes into a new M-phase arrest, known as metaphase III (MIII) (Susko-Parrish et al., 1994; Collas et al., 1995). This phase can be circum-

vented by additional treatments that inhibit protein synthesis, such as protein phosphorylation (6-DMAP) (Susko-Parrish et al., 1994). In our experiment, rabbit MII oocytes treated with electroporation followed by inositol 1,4,5-trisphosphate plus 6-DMAP resulted in high activation and parthenogenetic development rates. Electrical pulses induced obvious ionic calcium influx, while IP3, a widespread Ca^{2+}-release agonist, binds to IP3 receptors (IP3R) localized in the endoplasmic reticulum (ER) and induces Ca^{2+} release (Miyazaki et al., 1993). Perhaps EID treatment, which stops protein synthesis as well as phosphorylation induced more complete activation leading nonreversable reduction of MPF (Liu et al., 1998a,b; Liu and Yang, 1999) and resulted higher cleavage and development rate in EID group. Our findings also indicate that blastocyst apoptotic index is significantly lower in EID than that in ID group or ED group. The results demonstrate that EID treatment used for rabbit oocyte activation significantly improved cleavage, blastocyst development and total cell number, strikingly decreased apoptosis level of blastocyst. Thus, we used the similar activation protocols to induce the activation of rabbit NT embryos derived from fresh cumulus cells. In our experiment, analysis of different treatment times with 6-DMAP for rabbit reconstructured embryo were tested by comparing the cleavage rate, blastocyst development, cell number and apoptosis (Table 2). Our results demonstrated that treatment of NT embryos using EID (3 hr) significantly improved blastocyst development rate and cell number than exposing embryos to 6-DMAP for 2 or 4 hr, and this treatment produced blastocysts with similar cell number to that from the fertilized blastocyst (our unpublished results) and with markedly lower apoptotic index than that in EID (4 hr) group. The lower blastocyst developmental rate and cell number in EID (4 hr) group may be associated with cell death by apoptosis. DMAP treatment of the electrofused oocyte-cell complexes might affect the reprogramming process of the donor nuclear material. Prolonged exposure to 6-DMAP resulted in chromosomal abnormalities in cattle (De la Fuente and King, 1998; Van de Velde et al., 1999), while apoptosis may play an important role in eliminating the cells with the abnormal chromosomes (Hardy, 1997). Thus, 4 hr DMAP treatment on the NT rabbit embryo may result in the lower cell number because of higher level of apoptosis. Treatment the NT embryos with 2 hr DMAP resulted in markedly lower blastocyst development rate and cell number than that in 3 hr DMAP group, suggesting that 3 hr DMAP treatment is more efficient for embryo development. We postulate that this modified procedure (EID, 3 hr) may enhance complete nuclear reprogramming and therefore improve the developmental capacity of the nuclear-transplanted eggs.

Fluctuations in $[Ca^{2+}]$i are known to mediate a very large array of cell functions including chromosome motion (Zhang et al., 1990), cycle regulation (Hepler, 1989), extensive changes in nonhistone chromosomal proteins (Stros et al., 1994), chromatin configuration (Dobi and Agoston, 1998), and gene expression (Dolmetsch et al., 1997, 1998; Li et al., 1998). Such nonspecificity of the Ca^{2+} signal and its influence on a large array of interconnected and synchronized functions complicates comprehension of the mechanism by which small variations in $[Ca^{2+}]$i or their temporal sequencing influence the developmental processes at later stages. In mammalian eggs different patterns of Ca^{2+} increase during oocyte activation have been shown to drive activation and have an effect on the preimplantation development of the embryos (Ozil, 1990; Vitullo and Ozil, 1992; Collas et al., 1995; Bos-Mikich et al., 1997). Ozil and Huneau (2001) found that the amplitude, the number and the time interval of Ca^{2+} influx during oocyte activation affect postimplantation development of rabbit. They hypothesized that the Ca^{2+} treatment, in addition to activating the oocyte to resume the cell cycle, can interfere with the epigenetic reprogramming of the zygote genome. These epigenetic changes (epimutations) are transmitted through the division of embryo cells and result in the alteration of gene expression pattern (Ozil and Huneau, 2001). In our experiments, EID treatment may influence the development and apoptosis of the blastocyst by Ca^{2+} fluctuations. We proposed that Ca^{2+} influx during oocyte activation may interfere with the genes expression of pro- and anti-apoptosis in preimplantation embryos.

In conclusion, our results demonstrate that EID treatment is an efficient procedure to induce rabbit oocyte activation and this treatment can increase blastocyst rate and total cell number, while decrease the blastocyst apoptotic index. Treatment of rabbit NT embryo with EID (3 hr) results in higher blastocyst development rate, cell number, and lower apoptotic level. Small changes in the Ca^{2+} signal is likely to result in a strong impact on the immediate oocyte response (activation), later development and apoptosis of rabbit embryos.

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