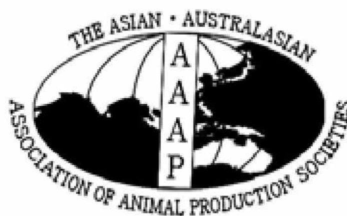

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Effect of Oocyte Activation Regimens on Ploidy of Nuclear Transfer Embryos Reconstructed with Fetal Fibroblasts in Rabbit

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ABSTRACT : Considerable attention has been focused on the cloning of mammalian embryos, as a consequence of poor development, in order to enhance the application of genetic engineering. Experiments were conducted to compare the developmental competence of parthenotes and reconstructed (NT) rabbit eggs with fetal fibroblasts (FFs) following various activation regimens. Oocytes and NT eggs were exposed to: electric stimulation (EST, Group 1) and EST followed by 6-dimethylaminopurine (DMAP, Group 2), cycloheximide (CHX, Group 3) or DMAP/CHX (Group 4). Pronuclear (PN) status, cleavage, blastocyst development and the ploidy were assessed. In parthenote groups 1, 2, 3 and 4, the PN formation differed significantly. And, the cleavage and blastocyst rates were 41.7 and 5%, 75.6 and 53.7%, 68 and 36%, 82.1 and 52.6%, respectively, among treatments. Polyploidy was observed in 17.2% of EST plus DMAP and 44.9% of EST plus DMAP/CHX groups. In SCNT groups (Group 1, 2, 3 and 4), the cleavage and blastocyst rates were 28.6 and 7.1%, 58.3 and 29.2%, 56.8 and 24.1%, 64.5 and 27.8%, respectively. The chromosomal composition differed significantly ($p<0.05$) among treatments. In Group 2 and 3, 53.8% and 81.8% of embryos revealed diploid chromosomal sets, respectively. However, in Group 4, 53.3% of embryos showed abnormal ploidy (mixoploid). Although DMAP or combination with DMAP/CHX resulted in higher *in vitro* development of rabbit SCNT embryos, higher incidence of chromosomal abnormality may induce problems related to fetal loss of at late stage of development. (**Key Words** : Activation, Nuclear Transfer, Development, Ploidy, Rabbit)

INTRODUCTION

Since the first report of cloned rabbit with somatic cell nuclear transfer (SCNT) by Chesne et al. (2002), numerous studies have been performed to improve the developmental competence and efficiency of cloning (Cervera and Garcia-Ximenez, 2004; Li et al., 2006). However, the *in vivo* viability of rabbit SCNT embryos is still in its infancy. The success of animal cloning by SCNT depends upon many factors including donor cell cycle, oocyte quality, SCNT procedure, and oocyte activation. Among these factors, oocyte activation is one of the critical components (Kim et al., 2005; Bhak et al., 2006).

The most important aspect in oocyte activation would be to create the transient Ca^{2+} oscillations and maintain a low level of maturation promoting factor (MPF) and mitogen activated-protein (MAP) kinase activities in the MII-arrested recipient oocyte (Moos et al., 1995). In bovine oocytes. To enhance the embryo development into later stage, eggs were achieved by combined treatments of Ca^{2+} ionophore with inhibitors of protein synthesis or phosphorylation, such as 6-dimethylaminopurine (DMAP) or cycloheximide (CHX) (Rho et al., 1998; Wells et al., 1999). Both DMAP and CHX, broad-spectrum inhibitors, non-specifically affect several metabolic pathways in oocytes and consequently may impair further embryonic development.

Activation of rabbit oocytes could be achieved by single or multiple electric DC pulse resulting in pronuclear formation and subsequent development to preimplantation embryos (Onodera and Tsunoda, 1989; Ozil, 1990; Yin et al., 2000). Although combined treatment of calcium stimulation with DMAP and CHX induced enhanced *in vitro* development, the overall efficiency of the success in SCNT is still too low (1.6%) (Yin et al., 2000; Dinnyes et al., 2001; Chesne et al., 2002; Liu et al., 2004; Liu et al., 2005), causing to presumably, inadequate reprogramming of differentiated somatic nuclei and followed by chromosomal aberrations (Bhak et al., 2006). The structural composition of SCNT embryos should also be considered as another factor responsible for the developmental obstacles since the

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formation of developmentally competent blastocyst involves the processes of cell division and chromosomal composition. It is important to note that development of chromosomally abnormal embryos is retarded compared to those of normal diploid embryos (Slimane-Bureau and King, 2002; Bureau et al., 2003). Particularly, the incidence of chromosomal aberrations is higher in SCNT embryos than IVF embryos (Yoo et al., 2003).

However, there is no report on direct comparisons, including developmental capacity and chromosome aberrations, of the efficiency of rabbit SCNT embryos cloned with FFs by different activation treatments. Therefore, with the aim of producing diploid NT embryos, the effects of phosphorylation or protein synthesis inhibitors were assessed for NT embryos with fetal fibroblasts on subsequent developmental ability and ploidy.

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Company (St. Louis, MO) and media from GIBCO BRL, unless otherwise specified. The medium used for oocyte flushing was Dulbecco's modified PBS (DPBS) supplemented with 3 mg/ml of bovine serum albumin (BSA, Fraction V) and 1% (v/v) penicillin-streptomycin (10,000 IU and 10,000 μg/ml, respectively; Pen-Strep). CR1aa (114 mM NaCl, 3.1 mM KCl, and 26.2 mM $NaHCO_3$) medium (Rosenkrans et al., 1993) containing 0.15 mg/ml L-glutamine, 0.55 mg/ml L-lactate, 10 μl/ml non-essential amino acids solution (NEAA) (100×), 20 μl/ml essential amino acids solution (EAA) (50×), 2.5 mM sodium pyruvate and 3% BSA (Essential fatty acid free, Fraction V). Fetal fibroblasts were incubated in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 1% (v/v) Pen-Strep. Tyrode's albumin lactate pyruvate HEPES (HEPES-TALP) (114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.4 mM $NaH_2PO_4H_2O$, 2 mM $CaCl_2$ $2H_20$, 0.5 mM $MgCl_26H_2O$, and 10 mM Na lactate) medium containing 2 mg/ml BSA and 10 mM HEPES was used for micromanipulation.

Establishment of fetal fibroblasts

Fetal fibroblasts (FFs) were obtained from 16 dpc (days post coitum) rabbit fetuses. Each fetus after decapitation and evisceration was washed in DPBS and then placed into 1 ml of 0.05% Trypsin-EDTA solution for 15 min. After adding of 1 ml of DPBS supplemented with 10% FCS, the cells were suspended by aspirating through an 18G needle attached to a 10 ml syringe. After twice centrifugation at 350×g for 10 min each in DMEM, the cell suspension was transferred in to 0.1% gelatin pre-coated 60 mm tissue culture dishes containing 4 ml of fibroblast cell culture medium. Cells were cultured at 39°C in a humidified atmosphere of 5% CO_2 in air with medium change every three days until the cells reached confluence. Cells were harvested by trypsinization using 0.05% Trypsin -EDTA solution and two parts were used for further passages while one part was frozen in 10% dimethylsulfoxide (DMSO) in culture medium and stored in liquid nitrogen until further use. For nuclear donor, cells at the 3-8 passages were grown to confluence, trypsinized and resuspended in HEPES-TALP medium.

Superovulation and oocyte collection

New Zealand White rabbits were housed in an environmentally controlled room with a 12.00 h dark: 12.00 h light cycles and given free access to laboratory chow and water. For oocyte collection, 6 week-old rabbits were superovulated as described by Kanayama et al. (1994), with slight modifications. Briefly, each donor received a single subcutaneous injection of 40 mg FSH (Folltropin®-V, Vetrepharm, Canada) dissolved in 1 ml of 25% polyvinylpyrrolidone (PVP, MW 360,000) solution. At 40 h post-FSH injection, 1 ml of $PGF_2\alpha$ (Lutalyse, Upjohn) was administered intramuscularly (i/m). Ovulation was induced at 20 h post $PGF_2\alpha$ administration with a single i/m injection of 100 IU hCG. Ovulated oocytes were collected at ~13.5 h post-hCG injection by surgical flushing of the oviducts with DPBS supplemented with 0.1 mg/ml polyvinylalcohol. Oocytes were freed off their cumulus cells by brief exposure to 200 IU hyaluronidase in DPBS and subsequent repetitive pipetting using a fine glass pipette (inner diameter of ~150-μm). Oocytes with a dense cytoplasm and first polar body were selected under a microscope at ×200 magnification for subsequent activation and nuclear transfer (NT) experiment.

Nuclear transfer (NT)

The oocytes were transferred to HEPES-TALP containing 5 μg/ml cytochalasin B for 10 min, and then into a HEPES-TALP drop for the process of NT. The first polar body and metaphase II plate with small volume of surrounding cytoplasm were removed by aspiration with a 20 μm internal diameter enucleation pipette. The enucleated oocytes were labeled with 0.5 μg/ml bisbenzimide (Hoechst-33342) for 2 min at room temperature in HEPES-TALP medium and ensured that the nucleus was completely removed. After being trypsinized, a single intact cell was transferred by micropipette into the perivitelline space of each enucleated oocyte.

For fusion of the cytoplast and donor cell, the egg was exactly oriented in BTX Electro chamber (BTX, Inc., San Diego, CA) filled with 0.28 M mannitol solution containing 0.01 μM $CaCl_2$ and 0.01 μM $MgCl_2$, and pulsed with 1.6 KV/cm DC for 60 (sec using a BTX Electro-Cell Manipulator 200. One hour after fusion, the eggs were re-

examined. Eggs in which fibroblast could be seen in the perivitelline space were discarded. Fused eggs were randomly assigned into 4 experimental groups as follows: cultured in 1.9 mM DMAP for 3 h (Group 1), in 10 μg/ml CHX/5 μg/ml cytochalasin B for 3 h (Group 2), in combination of 1.9 mM DMAP and 10 μg/ml CHX for 3 h (Group 4) and without activation (control).

Activation and culture

Oocytes pulsed electrically with 1.6 KV/cm DC for 60 μsec and SCNT eggs were randomly assigned to the 3 experimental groups. Oocytes not exposed further (Group 1, EST), exposed to 1.9 mM DMAP for 3 h (Group 2, EST+DMAP), 10 μg/ml cycloheximide for 3 h (Group 3, EST+CHX), and combined treatments of DMAP with CHX for 3 h (Group 4, EST+DMAP/CHX). All eggs were then cultured in sets of 10 in 30 μL drops of CR1aa medium and maintained for 7 days at 39°C in a humidified atmosphere of 5% CO_2 in air. On Day 3 (Day 0 = activation), the cultures was transferred in to CR1aa supplemented with 10% FCS instead of BSA. The rates of cleavage and blastocyst formation were assessed at 48 h and 108 h post activation (hpa), respectively.

Cytological analysis

Pronuclear (PN) formation of parthenotes at 18 h was assessed by being fixed in methanol-acetic acid (3:1) for overnight and followed by stained with 1% aceto-orceine. After being mounted onto a precleaned microscope slide, the pronuclei of the parthenotes were counted under a microscope.

At 108 h post-activation, cell number and chromosomal status of reconstituted embryos were assessed by the method as described by King et al. (1979). Briefly, embryos were synchronized at metaphase by culturing under condition of TCM containing 0.05 μg/ml KaryoMAX Colcemid® solution for 6 h. Embryos were subsequently transferred into 1% sodium citrate solution for 4 min at room temperature. Single embryo in 0.5 μl of the sodium citrate solution and 1.5 μl of methanol:acetic acid (1:1, v/v) was placed on pre-cleaned microscope slide and gently blown by mouth to spread the chromosomes. After drying, slides were fixed in methanol:acetic acid (3:1, v/v) overnight and stained with 4% (v/v) Giemsa solution for 10 min. The nuclei were counted under a compound microscope (×200) and chromosome spreads were evaluated under oil-immersion optics (×1,000). Embryos were classified as being haploid, diploid, polyploid or mixoploid.

Experimental design

The present study comprised of four experiments on determining the developmental rates and ploidy of parthenotes and SCNT embryos by different activation treatments. Oocytes and SCNT eggs were assigned into 4 treated groups. Group 1 (electric stimulation, EST): Oocytes and SCNT eggs were electrically activated. Group 2 (EST+DMAP): oocytes and SCNT eggs were activated electrically and followed by culture in 1.9 mM DMAP for 3 h or 10 μg/ml CHX for 3 h (Group 3, EST+DMAP). In Group 4 (EST+DMAP/CHX), oocytes and SCNT eggs were electrically activated and followed by culture in the combined mixtures of DMAP and CHX for 3 h. In Experiment 1, PN formation of parthenotes produced by 4 treated groups was assessed (Figure 1). In total, 193 eggs were used in three replicates. In Experiment 2, parthenotes produced by the three different activation treatments were compared of the rates of cleavage and development to blastocyst. In Experiment 3, SCNT embryos produced by the same activation treatments to parthenotes were compared for the rates of cleavage and development to blastocyst. Finally, in experiment 4, SCNT embryos produced at 108 h post activation by three different activation treatments were compared for their ploidy (Figure 2). A total of 39 SCNT embryos were analyzed.

Statistical analysis

Differences among treatments were analyzed using one-way analysis of variance (ANOVA) after arcsine transformation of the proportional data of PN formation, cleavage, development, and ploidy. Comparisons of means among treatments were performed using the Turkey-Kramer multiple comparisons test. For chromosome analysis, differences among treatments were evaluated by the chi-square test. Differences were considered significant when $p<0.05$.

RESULTS

General observations

In preliminary experiments, the efficiency of superovulation by multiple (six consecutive) injections of FSH, the most common method used in rabbit was compared to single injection of FSH dissolved in PVP solution. The mean number of oocytes recovered by single FSH injection did not differ from that by multiple FSH injection. Further, no significant difference between single and multiple injections was observed in the oocyte quality based on the cytoplasm density and maturation (MII) rate (data not shown). Therefore, single FSH injection was used for superovulation in subsequent trials. At the time of cumulus cell removal, approximately 90% of the oocytes had a visible first polar body and uniformly dense ooplasm, making them suitable for further experiments.

Table 1. Activation and pronuclei formation of oocytes at 18 h post-activation following different activation treatments

Group (treatment)	Oocytes	Activated oocytes (%)				
		Total	1PN	2PN	≥3PN	Un-activated
1 (EST)	44	33 (75.0)[a]	33 (75.0)[a]	-	-	11 (25.0)[a]
2 (EST+DMAP)	47	38 (81.0)[a]	4 (8.5)[b]	26 (55.3)[a]	8 (17.2)[a]	9 (19.1)[a]
3 (EST+CHX)	53	43 (81.1)[a]	41 (77.4)[a]	2 (3.8)[b]	2 (3.8)[b]	8 (15.1)[a]
4 (EST+CHX/DMAP)	49	44 (89.5)[a]	2 (4.1)[b]	18 (36.7)[c]	22 (44.9)[c]	5 (10.2)[a]

[a-c] Percentages with different superscripts within columns are significantly different ($p>0.05$).

1PN: one pronucleus; 2PN: two pronuclei; EST: electric stimulation; DMAP: 6-dimethylaminopurine; CHX: cycloheximide.

Table 2. Cleavage and development of parthenotes subjected to different activation treatments

Group (treatment)	Oocytes	Development to (%)	
		Cleavage	Blastocysts
1 (EST)	60	25 (41.7)[a]	3 (5.0)[a]
2 (EST+DMAP)	82	62 (75.6)[bc]	44 (53.7)[b]
3 (EST+CHX)	75	51 (68.0)[b]	26 (36.0)[c]
4 (EST+CHX/DMAP)	78	64 (82.1)[c]	41 (52.6)[b]

[a-c] Percentages with different superscripts within columns are significantly different ($p<0.05$).

EST: electric stimulation.

DMAP: 6-dimethylaminopurine; CHX: cycloheximide.

3 Replicates

Table 3. Cleavage and development of NT embryos subjected to different activation treatments

Group (treatment)	Eggs	Development to (%)	
		Cleavage	Blastocysts
1 (EST)	42	12 (28.6)[a]	3 (7.1)[a]
2 (EST+DMAP)	48	28 (58.3)[bc]	14 (29.2)[b]
3 (EST+CHX)	58	33 (56.8)[b]	14 (24.1)[b]
4 (EST+CHX/DMAP)	79	68 (64.5)[c]	22 (27.8)[b]

[a-c] Percentages with different superscripts within columns are significantly different ($p<0.05$).

EST: electric stimulation.

DMAP: 6-dimethylaminopurine; CHX: cycloheximide.

3 replicates.

Effect of different activation treatments

At 18 h post-activation, the activation rate and PN formation of oocytes were determined based on the PN appearance as showed in Table 1. A range of 75.0-89.5% of oocytes in all four treated groups was shown to be activated, with no significant differences among the groups ($p<0.05$). However, the rate of PN number differed remarkably among groups. In groups 1 and 3, rate of one PN formation (>95%) was significantly ($p<0.05$) higher than those of groups 2 and 4 (10.5%, 4/38 and 4.5%, 2/44, respectively). The rate of 2 PN in group 2 was significantly ($p<0.05$) higher than those in groups 3 and 4 (55.3% vs. 3.8 and 36.7%, respectively). In particular, DMAP treated groups (groups 2 and 4) revealed significantly ($p<0.05$) higher abnormal PN formation than without DMAP treated groups (group 1 and 3).

Cleavage and development of parthenotes subjected to different activation treatments

The developmental capability of oocytes that had been activated by different activation treatments was compared. The results are summarized in Table 2, which shows that the rates of oocyte cleavage and development to blastocyst. In group 1 treated with EST alone resulted in 25 (41.7%) out of 60 oocytes to cleave, but only 3 (5.0%) developed into blastocyst stage. In contrast, cleavage rates of oocytes treated with EST plus DMAP or CHX in groups 2, 3 and 4 were significantly ($p<0.05$) increased to 75.6%, 68.0% and 82.1%, respectively. The rate of blastocyst in group 3 oocytes, which treated with EST plus CHX was 36% with significantly ($p<0.05$) lower than that in group 2 (53.7%). However, when DMAP and CHX were combined (group 4), development rate to blastocyst stage was markedly increased to 52.6%, but it did not differ compared to group 2.

Cleavage and development of NT embryos produced by different activation treatments

The rates of cleavage and development of embryos cloned with fetal fibroblasts produced by different activation treatments were compared. The results are summarized in Table 3. In group 1 eggs treated with EST alone for the fusion of couplet of ooplasm and fetal fibroblast cleaved 12 (28.6%) out of 42, and 3 (7.1%) of the eggs developed to blastocyst stage with significantly ($p<0.05$) lower than those in groups 2, 3, and 4. In contrast, cleaved NT eggs in groups 2, 3 and 4 were significantly ($p<0.05$) increased to 58.3%, 56.8% and 64.5%, respectively. The rates of blastocyst in groups 2, 3 and 4 were 29.2, 24.1 and 27.8%, respectively, and did not differ among groups.

Ploidy of SCNT embryos produced by different activation treatments

The results of ploidy of SCNT embryos at 108 h post-activation produced by different activation treatments are presented in Table 4. A total of 311 chromosome spreads from 39 SCNT embryos in groups 2, 3 and 4, except group 1 embryos where few embryos developed, were analyzed to categorize them as diploid, polyploidy or mixoploidy. The rates of diploid in group 2 were significantly ($p<0.05$) higher than those in groups 2 and 4 (81.8% vs. 53.5 and 46.7%, respectively). In groups 2 and 4, the frequency of abnormality in which embryos were polyploidy and

Table 4. Ploidy of SCNT embryos at 108 h post-activation following different activation treatments

Group (treatment)	Embryos	Ploidy (%)		
		Diploidy	Polyploidy	Mixoploidy
2 (EST+DMAP)	13	7 (53.8)[a]	2 (15.4)	4 (30.8)[a]
3 (EST+CHX)	11	9 (81.8)[b]	-	2 (18.2)[b]
4 (EST+CHX/DMAP)	15	7 (46.7)[a]	-	8 (53.3)[c]

[a-c] Percentages with different superscripts within columns are significantly different (p>0.05).
EST: electric stimulation; DMAP: 6-dimethylaminopurine; CHX: cycloheximide.

mixoploidy was significantly ($p<0.05$) higher than those in group 3 (46.2%, 6/13 and 53.3%, 8/15 vs. 18.2%, 2/11, respectively).

DISCUSSION

Animal cloning by SCNT has emerged as an intensive field of research which holds much promise for understanding gene expression profiles, nuclear-cytoplasmic contributions to cellular function, improving the productivity of enormously valuable livestock, therapeutic applications with embryonic stem cells, and transgenic technology (Wells et al., 1999; McCreath et al., 2000; Keefer, 2004). SCNT offspring in several species provide certain evidence that nuclei from somatic cells, which were differentiated and grown in culture inserted into recipient enucleated ooplasm, have the ability to be reprogrammed. However, aberrant epigenetic reprogramming of the donor genome is still remained an essential issue in SCNT despite recent improvements (Wilmut et al., 1997; Wakayama et al., 1998; Chesne et al., 2002; Zhou et al., 2003). During the decades following the first success with cloning in domestic animal (Willadsen, 1986), tremendous effort was placed on improving the efficiency of the embryo cell cloning procedure. The development of NT by using somatic cells grown in culture has provided a new impetus to an alternative approach to human gene therapy. Despite the continuous advancement in NT procedures, the efficiency of overall cloning process still remains problematic. It has been suggested that at least in part on the appropriate combination of cell-cycle stages between the donor and host cell, and activation of recipient oocytes are one of the critical components of the current nuclear transplantation regimen (Stice and Robl, 1988). However, the absence of a paternal gamete in mammalian parthenotes appeared to limit their embryonic development. Successful embryo biotechnology procedures, including nuclear cloning and transgenesis, require an understanding as well as *in vitro* control of this activation phenomenon.

For SCNT, the oocyte must be stimulated to initiate the resumption of meiosis after nuclear donor injection. Maintenance of ploidy is very important to produce normal offspring. In this experiment, we conducted comparisons of pronuclear formation and *in vitro* development following various oocyte activation treatments. The influx of extracellular calcium by EST induced normal haploid, however *in vitro* development of parthenotes and to 2-cell stage and blastocyst was significantly poor than other combination treatments with EST plus various chemicals. The rise in intracellular calcium after fertilization or artificial calcium stimulation initiates a cascade of signaling events that result in the destruction of cyclin B1 by ubiquitin-dependent proteolytic degradation, and in the absence of cyclin B1, MPF activity decreased (Moos et al., 1995). It has been reported that EST could alone induce pronuclear formation, however, further development was compromised when compared to that resulted from combining electrical and chemical activation (Liu et al., 2005). Based on the results, although oocytes activated with EST alone formed one pronucleus, it could not support efficient *in vitro* development to blastocyst. This indicates that biochemical inhibitors are required for efficient oocyte activation in rabbit.

There are several related-kinases to intracellular calcium rise, of which MPF is inactivated by calcium. 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 metaphase arrest, known as metaphase III (MIII) (Susko-Parrish et al., 1994; Collas et al., 1995). However, further incubation with protein synthesis or phosphorylation inhibitor is necessary for the development of a pronucleus (Susko-Parrish et al., 1994). Such non-specificity of Ca^{2+} signal and its function on a large array of interconnected and synchronized functions complicates comprehension of the mechanism of its influence on the developmental processes at later stages (Liu et al., 2005). In the present study, the combination of protein synthesis inhibitors, and phosphorylation with EST had significantly higher *in vitro* development rate than EST alone treatment, indicating that these biochemical inhibitors support embryo development efficiently. However, in our parthenotes and SCNT results, DMAP and combination DMAP with CHX treatment induced severely high ploidy abnormality. DMAP inhibits protein kinases and consequently protein phosphorylation but not protein synthesis (Neant and Guerrier, 1988). DMAP may indirectly inhibit the activity of MPF allowing oocytes to drive into interphase of the first mitotic cell cycle, presumably as a uniform diploid (Susko-Parrish et al., 1994). A great incidence of chromosomal abnormalities as

assessed by haploidy, mixoploidy and polyploidy in DMAP-treated embryos in bovine was reported (Liu and Yang, 1998; Rho et al., 1998). When DMAP was applied to bovine NT, greater extent of abnormal ploidy and embryonic mortality with congenital malformation were observed by chromosomal abnormalities (Slimane-Bureau and King, 2002; Yoo et al., 2003). DMAP seems to bypass the normal chromosome segregation and second polar body extrusion process and instead induces instant chromosome condensation, decondensation and pronuclear formation (Susko-Parrish et al., 1994; Liu et al., 1998). Based on the above observations, it is concluded that DMAP treatment of embryos can accelerate the development rate but also results in increased incidence of chromosomal abnormalities. However, recently it has been reported that treatment of rabbit NT embryo with electrical pulse plus inositol 1,4,5-triphosphate (IP_3) and DMAP results in higher blastocyst development rate, cell number and lower apoptotic level (Liu et al., 2005). This procedure was postulated to enhance reprogramming process of the donor nuclear material and therefore improved the developmental capacity of the nuclear transplanted eggs.

CHX, a protein synthesis inhibitor, not only induced depletion of proteins maintaining MPF activity, but also prohibited the translation of cytoplasmic proteins controlling the initiation of DNA replication (Soloy et al., 1997). CHX combined with strontium acted synergistically to raise the efficiency of parthenogenetic activation rate than strontium alone in rat and mice (Bos-Mikich et al., 1995; Roh et al., 2003). But, the synergistic effect of combined treatment is unclear, because the inactivation of MPF and the successful completion of oocyte activation would generate signal(s) to stop the fertilization-induced Ca^{2+} oscillation at the proper stage (Deng and Shen, 2000). It has been suggested that the inhibition of protein synthesis prevents synthesis of cytostatic factor, which seems to be linked with MPF (Yang et al., 1994). A single Ca^{2+} peak induced by the electric pulse would destroy the existing cytostatic factor, whereas the following CHX treatment would prevent the renewal of cytostatic factor in the oocyte. These reduced levels of cytostatic factor may result in degradation of MPF. We found that oocytes were treated with a combination CHX and DMAP for 3 h for parthenotes and NT, induced higher activation rate than other groups. This indicates that CHX for the stabilization of MPF at a low level revealed as a suitable treatment than DMAP on the normality of ploidy.

In conclusion, this study demonstrated that EST plus CHX is suitable for oocyte activation in terms of higher blastocyst rates and normal ploidy. Further studies are needed to ascertain the reprogramming patterns following different cell types and activation treatments.

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