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Post-Activation Treatment with Cytochalasins and Latrunculin A on the Development of Pig Oocytes after Parthenogenesis and Somatic Cell Nuclear Transfer

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ABSTRACT

The objective of this study was to determine the effect of post-activation treatment with cytoskeletal regulators in combination with or without 6-dimethylaminopurine (DMAP) on embryonic development of pig oocytes after parthenogenesis (PA) and somatic cell nuclear transfer (SCNT). PA and SCNT oocytes were produced by using *in vitro*-matured pig oocytes and treated for 4 h after electric activation with 0.5 μM latrunculin A (LA), 10.4 μM cytochalasins B (CB), and 4.9 μM cytochalasins D (CD) together with none or 2 mM DMAP. Post-activation treatment of PA oocytes with LA, CB, and CD did not alter embryo cleavage (85.8～88.6%), blastocyst formation (30.7～32.4%), and mean cell number of blastocysts (33.5～33.8 cells/blastocyst). When PA oocytes were treated with LA, CB, and CD in combination with DMAP, blastocyst formation was significantly (P<0.05) improved by CB+DMAP (42.5%) compared to LA+DMAP (28.0%) and CD+DMAP (25.1%), but no significant differences were found in embryo cleavage (77.5～78.0%) and mean blastocyst cell number (33.6～35.0 cells) among the three groups. In SCNT, blastocyst formation was significantly (P<0.05) increased by post-activation treatment with LA+DMAP (32.9%) and CD+DMAP (35.0%) compared to CB+DMAP (22.0%) while embryo cleavage (85.5～85.7%) and blastocyst cell number (41.1～43.8 cells) were not influenced. All three treatments (LA, CB, and CD with DMAP) effectively inhibited pseudo-polar body extrusion in SCNT oocytes. The proportions of oocytes showing single pronucleus formation were 89.6%, 83.9%, and 93.3%, respectively with the increased tendency (P<0.1) by LA+DMAP and CD+DMAP compared to CB+DMAP. Our results demonstrate that post-activation treatment with LA or CD in combination with DMAP improves pre-implantation development of SCNT embryos and the stimulating effect of cytoskeletal modifiers on embryonic development is differentially shown depending on the origin (PA or SCNT) of embryos in pigs.

(Key words : cytochalasin, embryonic development, latrunculin A, somatic cell nuclear transfer, pig)

INTRODUCTION

Since the first cloned animal Dolly has been produced by the somatic cell nuclear transfer (SCNT) in sheep (Wilmut *et al*., 1997), viable cloned animals have been successfully produced in cattle (Cibelli *et al*., 1998; Kato *et al*., 1998), goats (Baguisi *et al*., 1999), and pigs (Betthauser *et al*., 2000). SCNT embryos have been used for several purposes such as for multiplication of superior genotypes with high genetic value, conservation of endangered species, and an important biological model for basic research (Hochedlinger and Jaenisch 2006). Generally, pig shows similarities with human in terms of physiology and genomics. They have also importance in biomedical and disease model research compared to other animals (Polejaeva *et al*., 2000; De Sousa *et al*., 2002; Im *et al*., 2005). At present, the viability of SCNT embryos is very poor with an extremely low efficiency of cloned offspring production (Betthauser *et al*., 2000; Lai *et al*., 2002). Therefore, it is crucial for practical application of SCNT technique to develop an efficient system to improve developmental competence of

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SCNT embryos.

Post-activation treatment is one of the important steps that directly affect the developmental competence of SCNT embryos (Machaty *et al*., 1999; De Sousa *et al*., 2002; Kurome *et al*., 2003; Miyoshi *et al*., 2005). As oocytes fertilized by sperm, reconstructed oocytes produced by SCNT tend to expel a portion of the somatic cell chromosomes in the form of a pseudo-polar body (PPB) when they are activated by electric or chemical stimuli (Lai *et al*., 2002). Loss of nuclear chromosomes from the reconstructed oocytes may result in nuclear aneuploidy, which in turn causes failure in normal embryonic development to term and decreases production efficiency of cloned animals (Kim *et al*., 2005; Russell *et al*., 2005; Song *et al*., 2009). Thus, an efficient method to inhibit PPB extrusion from the reconstructed oocytes is needed for maintaining normal nuclear ploidy of SCNT embryos. For this purpose, several chemicals such as cytochalasin, nocodazole, and colcemid have been applied for PA and SCNT oocytes in pigs but the results are contradictory depending on the chemicals used (De Sousa *et al*., 2002; Lee *et al*., 2004; Miyoshi *et al*., 2005; Meena and Das, 2006; Song *et al*., 2009). Cytochalasin B (CB) has been widely used to prevent the extrusion of PB in PA or SCNT oocytes after activation based on its ability to depolymerize actin filaments (Wilmut *et al*., 1997; Lee *et al*., 2004; Meena and Das, 2006). It is known that another cytoskeletal modifier cytochalasin D (CD) is 10 times more potent in regulating actin filaments and has shown less cytotoxicity than CB (Cooper, 1987). Recently, Himaki *et al* (2012) reported that post-activation treatment with latrunculin A (LA) was superior to CB on embryonic development of SCNT oocytes in pigs. LA is a purified toxin from the Red Sea sponge *Latrunculia magnifica* and a member of the cytoskeletal inhibitors, which inhibit the polymerization of actin as well as CB (Coué *et al*., 1987). LA shows a similar effect with CB at a low density a little less than 10 to 20 times and the effect of LA on actin filaments are 10～100 times more potent than cytochalasins (Wakatsuki *et al*., 2001). Thus, LA may have lower cytotoxicity when it is used for post-activation treatment of reconstructed oocytes. A non-specific kinase inhibitor, 6-dimethylaminopurine (DMAP), has been widely used together with electric activation to induce oocyte activation *in bovine* and pig SCNT (Vichera *et al*., 2010; Cervera *et al*., 2010). In this study, it was hypothesized that the regulatory action of cytoskeletal modifiers during post-activation period on nuclear remodeling would be varied and therefore embryonic development of PA or SCNT embryos would be differentially influenced. To test this hypothesis, PA and SCNT oocytes were treated with LA, CB, and CD in combination with or without DMAP after electric activation and then embryonic development and nuclear status of SCNT oocytes were examined.

MATERIALS AND METHODS

1. Culture Media

All chemicals were purchased from Sigma-Aldrich (USA) unless otherwise stated. *In vitro* maturation (IVM) of immature pig oocytes was conducted in medium-199 (M-199) (Invitrogen, USA) supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml epidermal growth factor, 75 μg/ml kanamycin, and 1 μg/ml insulin. The *in vitro* culture (IVC) medium for embryo development was porcine zygote medium-3 (PZM-3) containing 0.3% (w/v) fatty acid-free bovine serum albumin (BSA) (Yoshioka *et al*., 2002).

2. Oocyte Collection and IVM

Ovaries were obtained from prepubertal gilts at a local abattoir. Follicular contents were aspirated from superficial follicles (3～8 mm in diameter), pooled into 15-ml conical tubes and allowed to settle as sediment. The sediment was placed in HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA; Bavister *et al*., 1983) and observed under a stereomicroscope; only cumulus-oocyte complexes (COCs) with more than three layers of compact cumulus cells were selected. After washing twice in TLH-PVA and once in IVM medium, a group of 50～60 COCs was placed into each well of a 4-well multi-dish (Nunc, Denmark) containing 500 ml IVM medium with 10 IU/mL human chorionic gonadotrophin (hCG; Intervet International BV, Holland) and 80 μg/ml follicle stimulating hormone (Antrin R-10, Kyoritsu Seiyaku, Japan). COCs were cultured at 39℃ in a humidified atmosphere of 5% CO_2. After 22 h in maturation culture, the COCs were washed three times in fresh hormone-free IVM medium and then cultured in hormone-free IVM medium for an additional 18～21 h.

3. Preparation of Donor Cells

Pig fetal fibroblasts were seeded in a four-well plate and grown in Dulbecco's modified Eagle's medium (DMEM) with the nutrient mixture F-12 (Invitrogen), which was supplemented with 10% (v/v) fetal bovine serum and 75 μg/ml kanamycin,

until a complete monolayer formed. Donor cells were induced to synchronized at the G0/G1 stage of the cell cycle by contact inhibition for 48～72 h. Cells of the same passage (Passages 3～8) were used in each replicate for the various treatments. A single cell suspension was prepared by trypsinization of cultured cells and resuspension in TLH containing 0.4% (w/v) BSA (TLH-BSA) before nuclear transfer.

4. Nuclear Transfer

The base medium for oocyte manipulation was calcium-free TLH-BSA containing 5 μg/ml cytochalasin B. After 40 h of maturation, cumulus cells were removed by repeated pipetting in 0.1% (w/v) hyaluronidase in hormone-free IVM medium. Denuded oocytes were incubated for 15 min in a manipulation medium that contained 5 μg/ml Hoechst 33342, washed twice in fresh medium, and then placed into a manipulation medium droplet that was overlaid with mineral oil. Metaphase II (MII) oocytes were enucleated by aspirating the first polar body (PB) and MII chromosomes using a 17-μm beveled glass pipette (Humagen, Charlottesville, VA), and enucleation was confirmed under an epifluorescent microscope. After enucleation, a single cell was inserted into the perivitelline space of each oocyte. Cell-oocyte couplets were placed on a 1-mm fusion chamber overlaid with 1 ml of 280 mM mannitol that contained 0.001 mM $CaCl_2$ and 0.05 mM $MgCl_2$. Membrane fusion was induced by applying an alternating current field of 2 V, 1 MHz for 2 sec, followed by two direct current (DC) pulses of 1.75 kV/cm for 30 μsec using a cell fusion generator (LF101; NepaGene, Japan). The oocytes were incubated for 1 h in TLH-BSA and examined for fusion prior to activation.

5. Activation of Oocytes

Reconstructed oocytes were activated by two pulses of a 1.2 kV/cm DC for 60 μsec in 280 mM mannitol that contained 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$. For PA, the oocytes with PBs at 44 h of IVM were activated using a pulse sequence identical to that used to activate SCNT oocytes. Following electrical activation, SCNT and PA embryos were cultured in IVC medium containing LA, CB, CD with or without DMAP (see the 'Experimental design') for 4 h at 39℃in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

6. *In Vitro* Culture of PA and SCNT Embryos

The SCNT and PA embryos were washed three times in fresh IVC medium, transferred into 30 μl IVC droplets under mineral oil, and then cultured at 39℃ in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 7 days. Cleavage and blastocyst formation were evaluated on Days 2 and 7, respectively, with the Day of SCNT or PA designated as Day 0. The total blastocyst cell count was performed using Hoechst 33342 staining under an epifluorescence microscope.

7. Examination of Nuclear Status of SCNT Oocytes

To evaluate the nuclear status of reconstructed oocytes, oocytes that were cultured for 9～10 h after activation were mounted onto glass slides, fixed for 5～10 min in 25% (v/v) acetic acid in ethanol. Fixed oocytes were then stained in a solution of 1% (w/v) orcein in 45% (v/v) acetic acid. The extrusion of the pseudo-polar body and the number of PN were assessed under a phase contrast microscope.

8. Experimental Design

In Experiment 1, IVM oocytes were electrically activated, treated for 4 h with 0.5 μM LA, 10.4 μM CB, and 4.9 μM CD, and their effects on PA embryonic development were examined. In Experiment 2, effects of post-activation treatment of PA oocytes by LA, CB, and CD in combination with 2 mM DMAP on embryonic development were determined. Effects of LA, CB, and CD with DMAP during activation on the nuclear status (PPB extrusion and pronuclear formation) and embryonic development of SCNT oocytes were examined in Experiments 3 and 4, respectively.

9. Statistical Analyses

All statistical analyses were performed using the Statistical Analysis System (version 9.1; SAS Institute, USA). Data were analyzed using a general linear model followed by the least significant difference mean separation procedure when treatments differed at $P<0.05$. Percentage data were arcsine transformed before analysis to maintain homogeneity of variances. The results are expressed as the mean±standard error of the mean (SEM).

RESULTS

1. Effect of Post-activation Treatment with LA, CB and CD on *In Vitro* Development of PA Embryos

As shown in Table 1, post-activation treatment of PA oocytes with LA, CB, and CD did not alter embryo cleavage (85.8～88.6%), blastocyst formation (30.7～32.4%), and mean

Table 1. Effect of post-activation treatment with latrunculin A (LA), cytochalasins B (CB), and cytochalasins D (CD) on *in vitro* development of parthenogenetic pig embryos

Post-activation treatment	No. of embryos cultured	% of embryos developed to*		No. of cells in blastocyst
		≥2 cell	Blastocyst	
LA	195	85.8 ± 4.6	32.0 ± 4.7	33.8 ± 1.5
CB	191	88.6 ± 2.8	32.4 ± 3.2	33.5 ± 1.4
CD	183	87.4 ± 2.8	30.7 ± 4.2	33.6 ± 2.0

Five replicates.
* Percentage of the number of embryos cultured.

cell number of blastocysts (33.5～33.8 cells/blastocyst).

2. Effect of Post-activation Treatment with LA, CB, and CD in Combination with DMAP on *In Vitro* Development of PA Embryos

When PA oocytes were treated with LA, CB, and CD in combination with DMAP, blastocyst formation was significantly (P<0.05) improved by CB+DMAP (42.5%) compared to LA+DMAP (28.0%) and CD+DMAP (25.1%), but no significant differences were found in embryo cleavage (77.5～78.0%) and mean blastocyst cell number (33.6～35.0 cells/blastocyst) among the three groups (Table 2).

3. Effect of Post-activation Treatment with LA, CB, and CD in Combination with DMAP on *In Vitro* Development of SCNT Embryos

In SCNT, blastocyst formation was significantly increased by post-activation treatment with LA+DMAP (32.9%) and CD +DMAP (35.0%) compared to CB+DMAP (22.0%) while embryo cleavage (85.5～85.7%) and blastocyst cell number (41.1 ～43.8 cells) were not influenced (Table 3) by the treatment.

4. Effect of Post-activation Treatment with LA, CB, and CD in Combination with DMAP on Nuclear Status of SCNT Oocytes

All three treatments (LA, CB, and CD with DMAP) effectively inhibited pseudo-PB extrusion in SCNT oocytes. The proportions of oocytes showing single pronucleus formation were 89.6%, 83.9%, and 93.3%, respectively with the increased tendency (P<0.1) by CD+DMAP compared to CB+DMAP (Table 4).

DISCUSSION

Nuclear remodeling and reprogramming of donor nuclei in SCNT oocytes have utmost importance for normal embryonic development *in vitro* and *in vivo*. Activation of reconstructed oocytes greatly influences nuclear remodeling and reprogramming by regulating maturation promoting factor activity that is responsible for premature chromosome condensation and pronucleus formation. Thus, establishment of an efficient method to induce normal nuclear remodeling and reprogramming is needed to improve developmental competence of SCNT embryos. In this study, we examined the regulatory effect of various cytoskeletal modifiers such as LA, CB, and CD in

Table 2. Effect of post-activation treatment with latrunculin A (LA), cytochalasins B (CB), and cytochalasins D (CD) in combination with 6-dimethylaminopurine (DMAP) on *in vitro* development of parthenogenetic (PA) pig embryos

Post-activation treatment	No. of embryos cultured	% of embryos developed to*		No. of cells in blastocyst
		≥2 cell	Blastocyst	
LA + DMAP	118	77.9 ± 4.2	28.0 ± 3.7[a]	33.8 ± 2.1
CB + DMAP	124	78.0 ± 1.8	42.5 ± 2.8[b]	33.6 ± 1.8
CD + DMAP	122	77.5 ± 0.9	25.1 ± 2.7[a]	35.0 ± 2.9

Four replicates.
* Percentage of the number of embryos cultured.
[a,b] Within a column, values with different superscripts are different (P<0.05).

Table 3. Effect of post-activation treatment with latrunculin A (LA), cytochalasins B (CB), and cytochalasins D (CD) in combination with 6-dimethylaminopurine (6-DMAP) on *in vitro* development of somatic cell nuclear transfer (SCNT) pig embryos

Post-activation treatment	No. of embryos cultured	% of embryos developed to*		No. of cells in blastocyst
		≥2 cell	Blastocyst	
LA + DMAP	99	85.5 ± 3.7	32.9 ± 2.5[a]	43.8 ± 2.9
CB + DMAP	107	85.6 ± 3.9	22.0 ± 2.6[b]	41.1 ± 4.0
CD + DMAP	101	85.7 ± 1.5	35.0 ± 2.2[a]	43.7 ± 3.3

Five replicates.
* Percentage of the number of embryos cultured.
[a,b] Within a column, values with different superscripts are different (P<0.05).

Table 4. Nuclear status 9 h after activation of reconstructed oocytes that were treated with latrunculin A (LA), cytochalasins B (CB), and cytochalasins D (CD) in combination with 6-dimethylaminopurine (DMAP) for 4 h after electric activation

Post-activation treatment	No. of oocytes examined	Nuclear status (%)			
		1PN	1PN + PPB	2PN	Others
LA + DMAP	56	89.6 ± 1.9	0.0 ± 0.0	10.4 ± 1.9	0.0 ± 0.0
CB + DMAP	50	83.9 ± 0.6	1.9 ± 1.9	9.8 ± 0.8	4.4 ± 2.9
CD + DMAP	53	93.3 ± 6.7	0.0 ± 0.0	6.7± 6.7	0.0 ± 0.0

Three replicates.
PN, pronucleus, PPB, pseudo-polar body, others, intact nucleus.

combination with a protein kinase inhibitor DMAP on embryonic development and nuclear remodeling of pig oocytes after PA and SCNT. It was demonstrated that post-activation treatment with LA or CD in combination with DMAP improved pre-implantation development of SCNT embryos and the stimulating effect of cytoskeletal modifiers on embryonic development was differentially shown depending on the origin (PA or SCNT) of embryos in pigs.

Notwithstanding the different cytotoxicity and inhibitory actions on actin filaments (Cooper, 1987; Wakatsuki *et al*., 2001), LA, CB, and CD that were used for post-activation treatment did not show stimulatory or inhibitory effects on the embryonic development of PA oocytes. However, when PA oocytes were treated simultaneously with DMAP, CB showed increased blastocyst formation compared to LA and CD. This was consistent with the previous result (Grupen *et al*., 2002) that post-activation of PA oocytes with CB and DMAP increased blastocyst formation compared to single treatment with CB but was not consistent with another result that LA treatment during post-activation was more effective on preimplantation development of PA pig oocytes than CB. It was not known in this study how CB and DMAP could increase embryonic development. The doses and duration of treatment of LA and CD used in this study were adopted from the previous reports (Sugimura *et al*., 2008; Himaki *et al*., 2010) without optimization for IVM oocytes produced in this study. Supplementary studies would be needed to determine the optimal doses and treatment duration of each LA and CD. Interestingly, in contrast to the result shown in PA oocytes, treatment with CB and DMAP showed no stimulating effect on *in vitro* development of SCNT oocytes. Instead, blastocyst formation was increased by LA and CD but not CB, which was agreed with the previous result (Himaki *et al*., 2010). It was not clear the reason why PA and SCNT oocytes showed different responses to the same chemical treatment. The nuclear remodeling and reprogramming process in SCNT oocytes are quite different from that occurring in PA oocytes because the nuclei in SCNT oocytes are derived from differentiated somatic cells of G0/ G1 stage while PA oocytes have maternal chromosomes that are arrested at MII oocytes (Lee *et al*., 2004; Song *et al*., 2009). This difference in the nuclear status between PA and SCNT oocytes at the time of activation might be attributed

to the different responses to cytoskeletal modifiers. The proportion of SCNT oocytes formed single pronucleus after activation tended to increase by the treatment with LA and CD with DMAP, which was similar with the result of increased development in SCNT embryos by that treatment. As Song *et al* (2009) reported previously, formation of single pronucleus after activation may be an important factor for obtaining high developmental competence in SCNT embryos.

In summary, our results demonstrate that post-activation treatment with LA or CD in combination with DMAP improves preimplantation development of SCNT embryos. In addition, it is suggested from the differential effect of cytoskeletal modifiers on embryonic development of PA and SCNT oocytes that activation protocol using cytoskeletal regulators should be optimized for each PA and SCNT embryo production system in pigs. Further studies are needed to determine whether SCNT embryos treated with various cytoskeletal regulators and DMAP show normal *in vivo* viability.

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