Nuclear Remodeling and *In Vitro* Development Following Somatic Cell Nuclear Transfer in Swine

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ABSTRACT

This study was conducted to investigate nuclear remodeling and developmental rate following nuclear transfer of fetal fibroblast cells, ear skin cells and oviduct epithelial cells into porcine recipient oocytes. To test parthenogenetic activation, oocytes were treated with a 6-dimethylaminopurine (6-DMAP), a single DC-pulse (DC), calcium ionomycin (ionomycin), DC+6-DMAP and ionomycin + 6-DMAP after *in vitro* maturation. For nuclear transfer, *in vitro* matured oocytes were enucleated, and donor cells were transferred into oocytes. Cloned embryos were fused and stimulated with 6-DMAP for 4 h and cultured *in vitro* for 6 days. Among treatments for parthenogenesis, the activation rate of DC+6-DMAP treatment was significantly higher than that of single treatment groups ($p<0.01$), except for DC treatment group. However, the difference was not significant in activation rate compared to other complex treatment groups. Nuclear swelling of the cloned embryos was initiated at 60 min after stimulation and increased afterwards. Fusion rates were not different among different donor cells. Cleavage rates of DC treatment groups were significantly higher than those of DC+6-DMAP treatment groups ($p<0.05$) in case that fetal fibroblast and ear cells were used for nuclear donor. The cloned embryos from developed to blastocysts in oviduct epithelial cell nuclear transfer with DC+6-DMAP treatment was significantly higher compared to those with DC only treatment ($p<0.05$). However, no blastocyst was developed from nuclear transfer of fetal fibroblast and ear cells regardless of activation treatments. Based on these results, a proper activation stimulation may be necessary to increase the activation rate and the development to blastocyst in cloned porcine embryos.

(Key words : Nuclear remodeling, Somatic cell, Stimulation, Blastocyst)

INTRODUCTION

Production of cloned mammals by nuclear transfer of somatic cells has been extended to apply to the genetic improvement of farm animals, rescue of endangered species, and production of transgenic animals for medical use and organ transplantation (Wilmut et al., 1997). The nuclear transfer technique is currently unreliable, because the production efficiency of normal offspring is low (Tsunoda and Kato, 2000).

Mammalian oocytes are arrested at the diplotene stage of the first meiotic prophase, the germinal vesicle (GV) stage *in vivo* (Willadsen, 1986). This is the stage where intense transcription of the decondensed genome enables accumulation of RNA for the period of oocyte maturation and early embryonic development. Resumption of meiosis in *in vitro* is observed in fully-grown oocytes when they are released from the inhibitory influence of their follicles (Bordignon and Smith, 1998) and cultured in suitable conditions.

Oocyte activation is a series of events triggered by the fertilization or artificial stimulations and is essential for the beginning of embryonic development. However, the mechanism of oocyte activation is still poorly understood. Calcium has an important role in the events of egg activation and early development. Factors influencing pig oocyte activation by electrical stimulation were evaluated by their effect on the development of parthenogenetic embryos to the blastocyst stage. Collas et al. (1992) and Campbell et al. (1993) reported that the stimulated oocytes were conducted to nuclear envelop

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breakdown (NEBD), premature chromosome condensation (PCC), reformation of nuclear membrane, formation of pronucleus (PN) and nuclear swelling. The most effective method, however, for the activation of porcine oocyte has not yet been established. Therefore, it is important to develop a more efficient activating method for porcine oocyte in order to obtain higher stimulation and development results after nuclear transplantation.

The objective of the present study was to evaluate the effect of artificial stimulations on pronuclear formation, nuclear status and cloned embryo development.

MATERIALS AND METHODS

Oocytes Collection and *In Vitro* Maturation

Porcine gilt ovaries were obtained from a local abattoir and stored at 35℃during transportation. After arriving at the laboratory, the ovaries were washed 3 times with warmed Dulbecco PBS (Oxoid Ltd., Basingstoke, Hampshire, U.K.) and then stored in a water bath at 35℃ before use. Cumulus oocyte complexes (COCs) were aspirated from ovarian follicles 38mm in diameter using a 10ml syringe fitted with an 18-gauge needle. Follicular fluid was collected in 50ml conical containers and left for 5 min at 35℃. Subsequently, the COCs were washed 3 times with HEPES-buffered Tyrode albumin lactate pyruvate medium (TL-HEPES) with 0.1% polyvinylalcohol (PVA). Only COCs with uniform cytoplasm and at least 3 layers of compact cumulus cells were selected for maturation. Oocytes were normally matured in groups of 50 per 0.5ml of maturation medium at 39℃ in an atmosphere of 5% CO_2 in air. The base maturation medium was TCM-199 supplemented with 10% pFF (porcine follicular fluid), 5㎍/㎖ FSH (Sigma, USA), 20ng/㎖ epidermal growth factor (EGF; Sigma, USA) during the first 22h of culture only, after which culture was continued in the base maturation medium without hormone supplementation for additional 22hr.

Treatment for Oocyte Activation

Following *in vitro* maturation, the oocytes were denuded of cumulus cells by repeated pipetting on a warm stage at 37℃. The denuded oocytes were washed 3 times in Ca^{++}-free TL-HEPES-PVA medium and then rinsed twice in activation medium (0.3M mannitol, 0.1mM Mg^{++} and 0.05mM Ca^{++}). For these experiments, the matured oocytes were divided into the 6 treatment groups as follows; group 1: no-treatment (control), group 2: 2mM 6-dimethylaminopurine (6-DMAP, Sigma) for 4h, group 3: a single DC pulse of 2.0kV/cm for 30μsec (DC), group 4: 5μM calcium ionomycin (ionomycin, Sigma) for 4min, group 5: DC pulse followed by 6-DMAP for 4h and group 6: 5μM ionomycin for 4min followed by 6-DMAP for 4h. In all experiment, activated oocytes were cultured in the medium for 6 days in an atmosphere of 5% CO_2 and 95% air at 39℃.

Evaluation of Nuclear Status

Oocytes with or without treatments were mounted on slides, fixed with acetic alcohol (1:3 v/v) for 48h and stained with 1% aceto-orcein. The rates of oocytes that had underwent germinal vesicle breakdown (GVBD) and matured to the MII were evaluated. Activation was considered to have occurred if the oocytes were in the stages of PCC and PN formation.

Production of Nuclear Transferred Embryos

After maturation, cumulus cells were removed by vigorous vortexing for 5min in TL-HEPES-PVA medium containing 0.1% hyaluronidase. Enucleation and injection were carried out in TCM-199, supplemented with HEPES, 0.3% BSA and 7.5mg/ml cytochalasin B at 38℃. The first polar body and adjacent cytoplasm was removed by squeezing method. Serum starved fetal fibroblast, ear or oviduct epithelial cells were used as donor cells and introduced into the perivitelline space of the enucleated recipient oocytes through the hole made during enucleation. The reconstructed oocytes were placed between 0.2mm diameter platinum electrodes, 3.2mm apart, in fusion and activation medium. The medium used for fusion and activation was 0.3M mannitol, supplemented with 0.1mM $CaCl_2$, 0.1mM $MgCl_2$ and 0.5mM HEPES. For fusion and activation, a single DC-pulses of 2.0kV/cm for 30μsec were applied, using a BTX-Cell Manipulator 200 (BTX, San Diego, CA, USA). After fusion, the reconstructed zygotes were treated with 2mM 6-DMAP for 4h before *in vitro* culture.

In Vitro Culture of Embryos

The embryos were washed and transferred into 500㎕ of NCSU-23 medium covered with mineral oil in a 4-well dish. The culture environment was 5% CO_2 in air at 38.5 ℃. The nuclear transferred oocytes were evaluated for cleavage on Day 2, and percent blastocyst on Day 6.

Statistical Analysis

All experiments were assessed by one-way ANOVA. When ANOVA indicated a significant effect ($p<0.05$), treatment differences were compared using Chi-square test.

RESULTS

The pronucleus formation of no- or activated oocytes was shown in Table 1. The results obtained for oocytes

Table 1. Pronuclear formation of parthenogenetically activated pig oocytes

	No. of oocytes* (%)			
	Treated	Activated	1PN	2PN
Control	50	5 (10.0)[a]	5 (10±2.7)[a]	-
6-DMAP	50	9 (18.0)[a]	9 (18±3.2)[a]	-
DC	100	87 (87.0)[c]	85 (85±9.8)[c]	2 (2±2.5)
Ionomycin	100	36 (36.0)[b]	32 (32±5.7)[b]	4 (4±2.7)
DC+6-DMAP	138	129 (93.5)[c]	122 (88±12)[c]	7 (5±4.2)
Ionomycin+6-DMAP	149	131 (87.9)[c]	124 (83±7.9)[c]	7 (5±2.1)

*; Three sets of replications; Results were expressed as mean ± standard deviation.
Control: no-treatment; 6-DMAP: 6-dimethylaminopurine; DC: direct current; PN: pronucleus.
[a,b,c] Values with different superscripts within each column are significantly different ($p<0.01$).

no-treatment was used as controls. The activation rate of a single treatment group was significantly higher than that of control group ($p<0.01$), except for a 6-DMAP treated group. A single treatment of 6-DMAP was no effect on parthenogenetic activation in porcine oocytes. Interestingly a single treatment of DC pulse was significantly higher activation rate compared to the single treatment with 6-DMAP or ionomycin ($p<0.01$). Complex treatment groups showed higher activation rate than control and single treatment groups ($p<0.01$), except for DC pulse group.

The nuclear status of the oocytes was shown in Figure 1 and 2. As shown in Fig. 1, the oocytes stimulated with a single DC pulse underwent PCC from 40 to 100min. And nuclear swelling was initiated at 60min and observed to be increasing afterwards. After 2hr, the nucleus of the most oocytes was entered swelling status. The nuclear status of the oocytes treated with a single DC pulse followed by 6-DMAP for 4hr was shown in

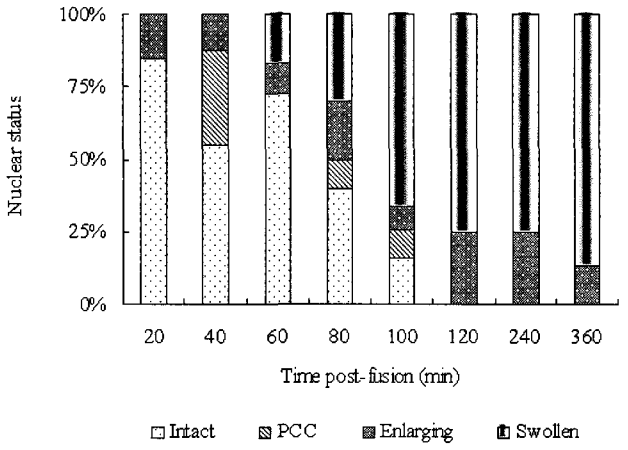

Fig. 1. The nuclear status of porcine nuclear transferred embryos after DC treatment. Intact: no morphological changes after stimulation; PCC: premature chromosome condensation.

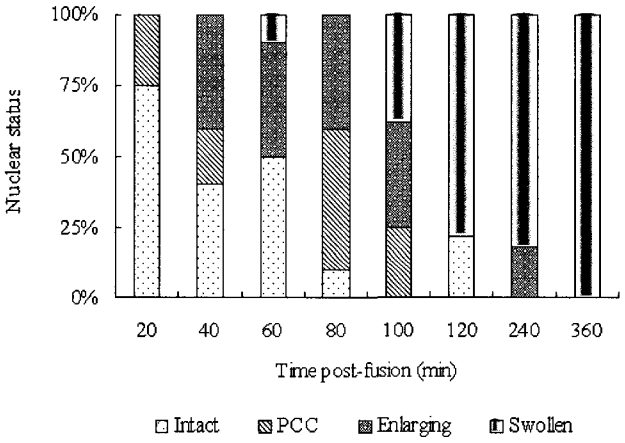

Fig. 2. The nuclear status of porcine nuclear transferred embryos after DC pulse followed by 6-DMAP treatment. Intact: no morphological changes after stimulation; PCC: premature chromosome condensation.

Fig. 2. The pattern of nuclear status in complex treatment group was similar to that of a single treatment group.

In vitro development of reconstructed oocytes using three different donor cells was presented in Table 2. The cleavage rate in a single DC treatment group was significantly high compared to that of the group treated with DC + 6-DMAP, when fetal fibroblast cell was used as donor cell ($p<0.05$). Using ear cells for donor nucleus, the group treated with a single DC pulse showed significantly higher in cleavage rate than that of the group treated with DC + 6-DMAP ($p<0.05$). However, the embryos developed to morulae and blastocyst was very low in both groups. There were no statistical significance in the cleavage rate between DC only and DC + 6-DMAP treated groups using oviduct epithelial cells as donor cells. However, the developmental rate to blastocyst in DC + 6-DMAP treatment group was significantly higher than that of the group treated with

Table 2. *In vitro* development of cloned embryos using three different donor cells

Donor cell	Stimulation	No. of embryos (%)			
		Fused *	Cleaved	M	BL
Fetal fibroblast	DC	56	39 (70±10)[b]	-	-
	DC+6-DMAP	59	31 (53±7.5)[a]	1 (1.6)	-
Ear	DC	53	38 (72±7.9)[b]	-	-
	DC+6-DMAP	48	24 (50±3.9)[a]	1 (2.0)	-
Oviduct epithelial	DC	55	41 (75±12)[b]	-	1 (1.8)[b]
	DC+6-DMAP	63	43 (68±5.6)[b]	1 (1.5)	8 (13±5.6)[b]

*; Three sets of replications; Results were expressed as mean±standard deviation.
DC: direct current; 6-DMAP: 6-dimethylaminopurine; M: morulae; BL: blastocyst.
[a,b;] Values with different superscripts within each column are significantly different ($p<0.05$).

a single DC pulse ($p<0.05$).

DISCUSSION

In this study, we investigated the effect of artificial stimulations on pronuclear formation, nuclear status of donor cell and cloned embryo development on swine. Recently, artificial stimulation to accelerate an increase of calcium in the ooplasm has demonstrated an improvement in the rate of oocyte activation (Hoshi et al., 1992). The mechanism, however, by which artificial stimuli generate oocyte activation is unclear. It has been suggested that increases in cytoplasmic calcium are important for the resumption of the cell cycle in all species examined (Whitaker and Patel, 1990; Miyazaki, 1991). Oocytes are normally arrested at metaphase of meiosis II at the time of ovulation and matured *in vitro*, due to cytostatic factor (CSF), which counteracts the activity of stabilized maturation promoting factor (MPF). Calcium elevation by artificial stimulation induces an inactivation or destruction of CSF, resulting in a resumption of meiosis event (Parrish et al., 1992).

The calcium elevating reagents such as ionomycin, Ca-ionophore or direct current (DC) has been used to stimulate oocyte activation and embryo development. When porcine oocyte are treated with a single treatment of DC, activation rate are 75 to 88% (Prather et al., 1991; Hagen et al., 1991; Wang et al., 1998). However, they reported that when they used other stimulators like Ca-ionophore, ionomycin or 6-DMAP one time, the activation rate are lower level. Our results are similar to other reports. A single pulse of DC is obtained significantly higher activation rate compared to a single treatment of ionomycin and 6-DMAP group. Except for DC treatment, complex treatments showed significantly higher activation efficiency on porcine oocytes. Especially DC + 6-DMAP treatment group showed highest oocyte activation rates compared to other treatment groups. One of the reasons to obtain higher activation efficiency using complex treatment is protein synthesis inhibitors such as 6-DMAP. The action of 6-DMAP is preventing protein phosphorylation (Jessus et al., 1991). The inhibitory effect was fully reversible for all inhibitors and the block seems to have stimulated meiotic resumption in different degrees according to the inhibitor (Beux et al., 2003).

We examined the nuclear remodeling after fusion and stimulation processes. The initiation of PCC was observed within 1h after fusion process. When nuclei were transferred to oocyte activated, NEBD and PCC were not observed (Cheong et al., 1994). Nuclear envelop breakdown and PCC are essential for the reprogramming of gene expression (Collas and Robl, 1991) and for the development of nuclear transferred embryos to term (Campbell et al., 1996). Maturation promoting factor is one of the most important factors to remodeling of the nuclear transferred embryos undergone NEBD, PCC, PN formation and nuclear swelling. In the present study, we examined the nuclear status of reconstructed embryo after stimulation. Premature chromosome condensation was observed from 40min in a single stimulation of DC pulse and from 20min in DC + 6-DMAP treatment group. The prolonged exposure of the donor nucleus to oocyte cytoplasmic factors after the loss of its nuclear envelop enhances the developmental potential of the reconstructed embryo (Szollosi et al., 1988). Even the nuclear status was similar to both treatment groups, the embryos developed to blastocyst was higher in complex treatment group than that of single stimulation group.

Based on these results, the complex treatment is effective method to obtain the higher oocyte activation and blastocyst development in porcine nuclear transplantation.

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