In Vitro Development and Chromosome Constitution of Porcine Parthenotes following Different Activation Treatments

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ABSTRACT

This study was conducted to examine the protein kinase inhibitors, 6-dimethylaminopurine (DMAP) and cycloheximide (CHXM) on the development and chromosome constitution of porcine parthenogenetic embryos. *In vitro* matured oocytes were activated by electric stimuli (ES) or a combination of ES with culture in 2 mM DMAP or 10 μg/ml CHXM for 4 hr. Activated oocytes were cultured in PZM-3 for 6 days. Some 1-cell embryos and blastocysts were fixed by air dry method to analyze the chromosome constitutions and/or total cell number. Blastocyst development of DMAP-treated group (26.7%) was significantly higher ($p<0.05$) than those of CHXM-treated and ES control groups. Ploidy in 1-cell stage embryos was not different among groups (77.3 to 81.0%), however, proportion of diploid chromosome constitutions was high in DMAP-treated group (61.9%, $p<0.05$). In the blastocyst stage, proportion of diploid chromosome plates was significantly high in DMAP-treated group (64.2%, $p<0.05$), and proportion of abnormal chromosome plates was higher in CHXM-treated group (36.6%, $p<0.05$) than DMAP-treated group (28.3%,). Proportion of embryos with abnormal chromosome constitutions was slightly increased by DMAP (40.0%) and CHXM (42.1%) treatment due to the increasing of mixoploid (47.4 and 52.0%). The present study shows that the DMAP treatment increase the development of porcine parthenotes. However, parthenogenetic activation by ES or combined treatment with ES and DMAP or CHXM detrimentally affects the chromosome constitutions of porcine parthenotes during early embryonic development, leads to increased abnormal ploidy in the blastocyst stage.

(Key words : Parthenogenetic activation, Ploidy, Electric stimulus, DMAP, Cycloheximide)

INTRODUCTION

Since the first clone sheep produced by a somatic cell nuclear transfer (SCNT) technique, numerous studies have been performed to produce clone pigs (Polejaeva *et al.*, 2000; Onishi *et al.*, 2000; Lai *et al.*, 2002; Yin *et al.*, 2002). However, the *in vivo* viability of pig SCNT embryos is still low. Animal cloning by SCNT depends upon many factors including nuclear transfer (NT) procedure and oocyte activation as well as nucleo-cytoplasmic interactions.

Production of parthenogenetic embryos (parthenotes) are valuable models for investigating the principles of oocyte activation, which may help to improve the efficiencies of current NT procedures.

Oocyte activation protocols were to oscillate of intracellular Ca^{2+} concentration in the oocyte cytoplasm by exposing the oocyte to an electric stimulus (Hagen *et al.*, 1991) or calcium ionophore (Funahashi *et al.*, 1994). Exposing the NT embryos (NTs) to electric pulses has been widely used not only for fusion of donor nucleus with oocyte cytoplasm but also for oocyte activation during NT procedure (Joliff and Prather, 1997). Moreover, it has been suggested that combined treatments of electric stimulus with a protein kinase inhibitor, DMAP (Susko-Parrish *et al.*, 1994) and with a protein synthesis inhibitor, CHXM (Presicce and Yang, 1994) enhance the developmental capacity of parthenotes. However, it is believed that the broad-spectrum nature of these activation compounds may have some unknown detrimental effects on embryo development that need to be elucidated. Some evidences suggest that these activation compounds may lead to inaccurate chromosome complement (ploidy) of the resulting parthenogenetic or SCNT embryos (Cha *et al.*, 1997; Winger *et al.*, 1997; Loi *et al.*, 1998; Mitalipov *et al.*, 1999; Hill *et al.*, 2000; Kim *et al.*, 2005; Alexander *et al.*, 2006). Therefore, this study was conducted to examine the effects of DMAP and CHXM on the development and

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chromosome constitutions of porcine parthenotes.

MATERIALS AND METHODS

In Vitro Maturation of Oocytes

Ovaries were obtained from a local slaughterhouse and transported to the laboratory in a 0.9% saline solution at 25~30℃. Cumulus-oocyte complexes (COCs) were collected by aspiration from ovary antral follicles (diameter, 3~6 mm) using an 18-gauge needle. The follicular fluid were pooled into 50 ml conical tubes and sediment was washed in Tyrode's lactate (TL)-Hepes containing 0.1% (w/v) polyvinyl alcohol (PVA, Sigma, St. Louis, MO, USA). The COCs with several layers of cumulus cells were selected and washed three times in maturation medium. For maturation culture, approximately 100~150 COCs were transferred into 500 μl of NCSU-23 medium supplemented with 0.6 mM cysteine (Sigma), 10 IU/ml PMSG (Sigma), and 10 IU/ml hCG (Sigma) and 10% (v/v) porcine follicular fluid (maturation medium), covered with paraffin oil and cultured for 22 hr at 39℃ in an atmosphere of 5% CO_2 in air. COCs were then cultured in maturation medium without hormone for 18~20 hr at 39℃ in an atmosphere of 5% CO_2 in air.

Parthenogenetic Activation

Following maturation, intact cumulus cells were removed by vortexing in the presence of 0.1% hyaluronidase. Oocytes that had extruded a first polar body were collect and placed between 0.2 mm diameter wire electrodes (1 mm apart) of a fusion chamber covered with 0.3 M mannitol solution containing 0.1 mM $MgSO_4$, 0.5 mM $CaCl_2$, and 0.1% (w/v) PVA. For parthenogenetic activation, a single DC pulse of 150 V/mm was applied for 30 μsec using a BTX Electro Cell Manipulator 200 (BTX, San Diego, CA, USA). After first activation treatment, oocytes were held in TCM-199 supplemented with 0.3% (w/v) BSA (Sigma) for 1 hr. Embryos were further activated at 1 hr after the first electric stimulation by exposure to two DC pulses of 100 V/mm for each 50 μsec, and treated with 2 mM 6-DMAP (Sigma) or 10 μg/ml CHXM (Sigma) for 4 hr before *in vitro* culture.

In Vitro Culture

Parthenotes were cultured in 50 μl droplets of PZM-3 supplemented with 3 mg/ml BSA covered with paraffin oil for 6 days under an atmosphere of 5% CO_2 in humidified air at 39℃. At days 2 and 6 of culture, cleavage and development to the blastocyst stage were examined, respectively.

Cytological Analysis

Some 1-cell stage embryos and all blastocysts were fixed by air-dry method to analyze their chromosome constitutions. Moreover, cell number in blastocysts was calculated using same method. One-cell stage embryos at 12 hr after activation and blastocysts were cultured in PZM-3 containing 3 mg/ml BSA and 3 μg/ml nocodazole (Sigma) for 12 hr and 8 hr, respectively. Embryos were then treated with 1% sodium citrate containing 5% FBS for 10 min before fixation. Embryos were fixed 2~3 min in 1st fixative (methanol : acetic acid : distilled water=5:1:4) and individual embryos were placed onto slides and spread by the dropping of 2nd fixative (methanol : acetic acid=3:1). After being air-dried, slides were placed in 2nd fixative for 10 min and further placed in 3rd fixative (methanol : acetic acid: distilled water=4:3:1) for 1 min. After being dried the slides were stained with 4% Giemsa solution for 10 min. Chromosome spreads and nuclei were counted under a compound microscope. Each spreaded chromosome plates from the one-cell and blastocyst stages were classified as being haploid, diploid, polyploid, or aneuploid. Blastocyst ploidy was determined from only the blastocysts showing multiple spreaded plates and classified as haploid, diploid, polyploid, or mixoploid.

Statistical Analysis

Percentages of cleavage and blastocyst development and cell number were analyzed by Duncan's multiple range test using the General Linear Models procedure in the Statistical Analysis System (SAS Institute, Inc., Cary, NC). Chromosome constitution was analyzed by Chi-square test.

RESULTS

Development of Parthenotes

The rates of cleavage, development, and total cell number of parthenotes produced by different activation treatments were summarized in Table 1. Cleavage rate did not differ among the different activation treatment groups (66.5 to 71.8%). Significantly high blastocyst formation rate (26.7%, $p<0.05$) was obtained in DMAP-treated group compared to ES control (18.5%) and CHXM-treated groups (19.3%). Total cell number in blastocysts were did not differ among the different activation treatment groups.

Ploidy of One-Cell Parthenotes

The ploidy of parthenotes produced by different activation treatments at the 1-cell stage was presented in Table 2. Normal ploidy in 1-cell stage embryos were not differ among three groups (77.3 to 81.0%), how-

Table 1. Development of porcine parthenotes produced by different activation treatments

Treatment*	No. of oocytes	No. (%) of embryos		Cell no. in blastocysts (Mean±SE)
		2-Cell	Blastocyst	
ES	265	156(66.5)	49(18.5)[a]	24.7±1.3
ES+DMAP	232	144(71.8)	62(26.7)[b]	26.3±1.3
ES+CHXM	332	201(67.6)	64(19.3)[a]	24.6±1.0

* ES, electric stimulation (one pulse of 150 V/mm for 30 μ sec and followed by two pulses of 100 V/mm for 50 μ sec, 1 hr after); ES+DMAP, combination of ES and DMAP culture for 4 hr; ES+CHXM, combination of ES and CHXM culture for 4 hr.

[a,b] Values with different superscripts differ significantly ($p<0.05$).

Table 2. Chromosome constitutions of one-cell stage porcine parthenotes produced by different activation treatments

Treatment*	No. of embryos examined	Chromosome constitution (%)**					
		Normal			Abnormal		
		N	2N	Total	≥ 3N	Aneuploid	Total
ES	50	18(36.0)[a]	22(44.0)[a]	40(80.0)	9(18.0)	1(2.0)	10(20.0)
ES+DMAP	42	8(19.0)[b]	26(61.9)[b]	34(81.0)	6(14.3)	2(4.8)	8(19.0)
ES+CHXM	44	11(25.0)[ab]	23(52.3)[ab]	34(77.3)	8(18.2)	2(4.5)	10(22.7)

* ES, electric stimulation (one pulse of 150 V/mm for 30 μ sec and followed by two pulses of 100 V/mm for 50 μ sec, 1 hr after); ES+DMAP, combination of ES and DMAP culture for 4 hr; ES+CHXM, combination of ES and CHXM culture for 4 hr.

** N, haploid; 2N, diploid; 3N, triploid.

[a,b] Values with different superscripts in the same column differ significantly ($p<0.05$).

Table 3. Chromosome constitutions of each metaphase plates from the blastocyst stage porcine parthenotes produced by different activation treatments

Treatment*	No. of metaphase plate analyzed	Chromosome constitution (%)**					
		Normal			Abnormal		
		N	2N	Total	≥ 3N	Aneuploid	Total
ES	91	12(13.2)	50(54.9)[a]	62(68.1)[ab]	29(31.9)	0	29(31.9)[ab]
ES+DMAP	106	8(7.5)	68(64.2)[b]	76(71.7)[a]	30(28.3)	0	30(28.3)[a]
ES+CHXM	82	9(11.0)	43(52.4)[a]	52(63.4)[b]	30(36.6)	0	30(36.6)[b]

* ES, electric stimulation (one pulse of 150 V/mm for 30 μ sec and followed by two pulses of 100 V/mm for 50 μ sec, 1 hr after); ES+DMAP, combination of ES and DMAP culture for 4 hr; ES+CHXM, combination of ES and CHXM culture for 4 hr.

** N, haploid; 2N, diploid; 3N, triploid.

[a,b] Values with different superscripts in the same column differ significantly ($p<0.05$).

ever, proportion of diploid chromosome constitutions was high in DMAP-treated group (61.9%, $p<0.05$) compared to ES control (44.0%).

Ploidy of Parthenogenetic Blastocysts

The ploidy of spreaded plates from parthenogenetic blastocysts produced by different activation treatments was presented in Table 3. Proportion of diploid chromosome plates was significantly high in DMAP-treated group (64.2%, 68/106, $p<0.05$) compared to ES control (54.9%) and CHXM-treated group (52.4%). Whereas, proportion of abnormal chromosome plates was higher

Table 4. Chromosome constitutions of porcine parthenogenetic blastocysts produced by different activation treatments

Treatment*	No. of** blastocyst	Chromosome constitution (%)***					
		Normal			Abnormal		
		N	2N	Total	≥ 3N	Mixoploid	Total
ES	23	3(13.0)	10(43.5)	13(56.5)	2(8.7)	8(34.8)	10(43.5)
ES+DMAP	25	1(4.0)	9(36..0)	10(40.0)	2(8.0)	13(52.0)	15(60.0)
ES+CHXM	19	2(10.5)	6(31.6)	8(42.1)	2(10.5)	9(47.4)	11(57.9)

* ES, electric stimulation (one pulse of 150 V/mm for 30 μ sec and followed by two pulses of 100 V/mm for 50 μ sec, 1 hr after); ES+DMAP, combination of ES and DMAP culture for 4 hr; ES+CHXM, combination of ES and CHXM culture for 4 hr.

** Ploidy was determined from only the blastocysts showing multiple metaphase plates.

*** N, haploid; 2N, diploid; 3N, triploid.

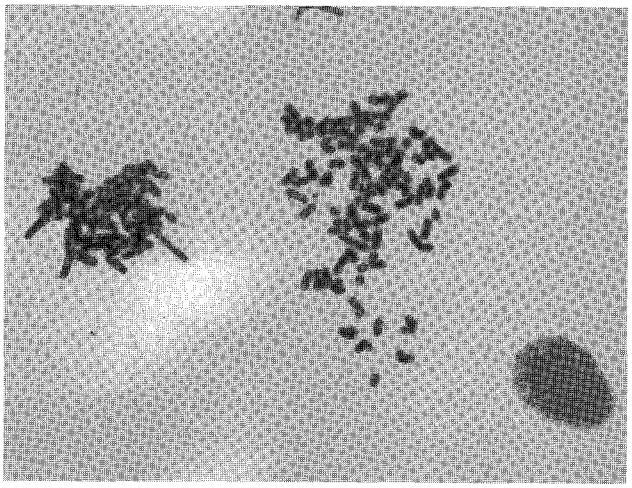

Fig. 1. Mixoploid complements of a parthenogenetic porcine blastocyst (40×).

in CHXM-treated group (36.6%, 30/82, $p<0.05$) than DMAP-treated group (28.3%, 30/106).

The ploidy of parthenogenetic blastocysts produced by different activation treatments was presented in Table 4. Proportion of embryos with normal chromosome constitutions (haploid or diploid) was slightly reduced by treatment with DMAP (40.0%, 10/25) or CHXM (42.1%, 8/19) due to the increasing of mixoploid (Fig. 1) compared to that of ES control (47.4 and 52.0% vs. 34.8%).

DISCUSSION

The induction of oocyte activation remains a challenge for successful SCNT embryo production. Among the intracellular Ca^{2+} oscillation induction methods, the combination of chemical compounds such as protein synthesis inhibitors (e. g., CHXM) or nonspecific kinase activity inhibitors (e. g., DMAP) have been used for the successful activation of mammalian oocytes (Presicce and Yang, 1994; Motlik *et al.*, 1998). Protein synthesis inhibitors usually restrict the synthesis or re-accumulation of cyclin B, thereby blocking the re-synthesis of MPF activity (Presicce and Yang, 1994). Whereas, nonspecific protein kinase inhibitors inhibit kinase activity of MPF (catalytic subunit CDK1) by inactivating MAPK (Motlik *et al.*, 1998; Gordo *et al.*, 2000). Therefore, persistent down regulation of MPF can be achieved by either of these chemical compounds for mammalian oocyte activation protocols.

In the present study, the development and chromosome constitutions of parthenotes were compared following activation with three different activation methods to find the effective activation method. The rate of blastocyst development in parthenotes activated with DMAP was greater than those in other treated groups. These finding is in agreement with previous studies, in which DMAP treatment of artificially activated oocytes increased the developmental potential of the parthenotes in cattle, mice and sheep (Susko-Parrish *et al.*, 1994; Leal and Liu, 1998; Alexander *et al.*, 2006). In mouse oocytes, DMAP arrested the extrusion the second polar body, resulting in formation of diploid parthenotes that were developmentally competent than haploid parthenotes (Szollosi *et al.*, 1993). However, the results in present study show that one-cell stage parthenotes had the similar chromosome constitutions in all groups. It has been suggested that the response to DMAP may vary according to the orientation and position of the spindle (Ledda *et al.*, 1996), which differs substantially in mouse and sheep oocytes (Thibault *et al.*, 1987).

In the present study, all three activation treated groups were linked to high frequencies of abnormal chromosomal complements throughout parthenogenetic development. Although higher proportions of oocytes displayed an expected chromosomal constitutions (ha-

ploid and diploid) at the 1-cell stage, a high proportion of abnormal chromosomal constitutions displayed in the blastocyst stage. This result has important implications for future genetic analysis of porcine parthenotes and may also provide some insight into the limited developmental potential (Winger *et al.*, 1997). There is a possible explanation for this observation. A centrosome in farm animal oocytes is absent. The centrosome is a paternally inherited structure as the sperm introduces the centrosome at fertilization in sheep (Crozet, 1993), rabbit (Pinto-Correia *et al.*, 1994), cattle (Long *et al.*, 1993), and human (Sathananthan *et al.*, 1991). The centrosome is responsible for organizing spindle poles and chromosome arrangement during the first cell cycle. Alexander *et al.* (2006) reported that SCNT embryos, which receive the centrosomes from their somatic cells, may have a superior ability in spindle pole orientation thus resulting in reduced rate of errors in chromosome segregation when compared to parthenotes. In addition, the broad-spectrum phosphorylation inhibition by DMAP may inhibit or permanently destroy some unknown kinases that are necessary for early nuclear remodeling and reprogramming (Alexander *et al.*, 2006).

In the previous studies in cattle (Winger *et al.*, 1997) and pig (Kim *et al.*, 2005), high proportion of abnormal ploidy, especially mixoploid, was appeared in the parthenogenetic blastocysts produced by a combination treatment of ethanol or electric stimulus with DMAP or CHXM. Kim *et al.* (2005) reported that DMAP accelerated mixoploidy in parthenogenetic pig embryos. In the present study, the proportion of mixoploid blastocysts was very high regardless of activation treatments, and the proportion of mixoploid was more high by combination with CHXM or DMAP. There was no difference between CHXM and DMAP. In the present study, we evaluated the ploidy of blastocysts just from the blastocysts with multiple metaphase plates.

In conclusion, the result of the present study shows that the DMAP treatment increase the development of porcine parthenotes. However, it is also suggested that parthenogenetic activation by ES or combined treatment with ES and DMAP or CHXM detrimentally affects the chromosome constitutions of porcine parthenotes during early embryonic development, leads to increased abnormal ploidy in the blastocyst stage.

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