Effects of Oocyte Maturational Age and Activation Conditions on the Development of Porcine Parthenogenetic Embryos

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ABSTRACT

This study was conducted to investigate the effects of oocyte maturational age and activation condition on *in vitro* development of porcine parthenogenetic embryos (parthenotes). Porcine follicular oocytes were matured *in vitro* for 30 to 44 hr. Maturation rate was examined during *in vitro* maturation (IVM) every 2 hr interval. The cdc2 kinase activity was measured at 36 and 44 hr of IVM. Some oocytes were activated at 36 or 44 hr of IVM by three different conditions; 1) single electric stimulation (1.5 kV/cm for 30 μsec; ES), 2) double electric stimulations (1.5 kV/cm for 30 μsec, followed by 1.0 kV/cm for 50 μsec after 1 hr; ES+ES) or 3) ES+ES followed by culture in 6-dimethlyaminopurine (6-DMAP) for 4 hr (ES+ES+D), and cultured for 6~7 days. Maturation rate was significantly increased as culture period was increased to 36 hr (66.9%, $p<0.05$), and then gradually increased to 87.1% at 44 hr of IVM. The cdc2 kinase activity was decreased ($p<0.05$) with culture period prolonged from 36 hr to 44 hr. Lower blastocyst formation rate (4.3%, $p<0.05$) were obtained by ES in 36 hr-matured oocytes compared to other treatments (16.5 and 20.5%) in the same age and the same treatment in 44 hr-matured oocytes (15.0%). High blastocyst formation rate (23.6%) was obtained by ES+ES+D in 44 hr-matured oocytes ($p<0.05$). These results demonstrate that porcine oocyte activation and *in vitro* development of parthenotes can be affected by interactions between oocyte maturational age and activation condition.

(Key words : Parthenogenesis, Maturational age, Activation condition, cdc2 kinase activity, Porcine oocytes)

INTRODUCTION

Since the production of a cloned lamb from somatic cells (Wilmut *et al.*, 1997), various species of animals have been cloned (Wakayama *et al.*, 1998; Baguisi *et al.*, 1999; Wells *et al.*, 1999; Polejaeva *et al.*, 2000). The production of transgenic or cloned animals would have great benefits in agricultural and human therapeutic applications. Nuclear transfer (NT) technique has been commonly used for the production of these animals, but its efficiency is still low. Several factors are considered to influence the developmental potential of NT embryos (NTs), including the maturational age of recipient oocytes, type and cell cycle stage of donor cells, activation of oocytes, reprogramming of a transferred nucleus, and so on.

A dynamic morphological and biological changes occur in oocyte cytoplasm when they are activated, such as resumption of meiosis, pronuclear formation, DNA synthesis, and progression of mitosis. These events are important for the early embryonic development which may be associated with oocytes quality. Most porcine oocytes reach the metaphase II (MII) stage by 36 hr of maturation culture (Kano *et al.*, 1998). In porcine NT studies, however, the oocytes cultured for about 44 to 48 hr have been usually used as a recipient cytoplasm (Koo *et al.*, 2000; Cheong *et al.*, 2002). During this oocyte arrest, cytoplasmic changes may occur, which might be responsible for the low efficiency of the NT procedure (Cheong *et al.*, 2000). Therefore, it is considered that aged oocytes may be unsuitable as a recipient oocyte having the ability to reprogram the donor nucleus (Cheong *et al.*, 2000). On the other hand, the aged oocytes can be easily activated, which might improve the development of reconstituted embryos, if they received the appropriate activation stimulus.

The activation stimulus is designed to mimic closely the events initiated by the sperm factor released upon fertilization and results in a Ca^{2+} rise in the treated oocyte (Saunders *et al.*, 2002). In order to activate the reconstructed embryos, several treatments such as electric pulse, chemical reagent or combination of both treatments have been used. Electrical pulse can induce several activation events very quickly involving calcium transient (Sun *et al.*, 1992) and pronuclear formation (Funahashi *et al.*, 1995). By these reasons elec-

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trical pulse has been used for the activation of the reconstructed embryos in NT procedures (Du *et al.*, 1995; Piotrowska *et al.*, 2000). In the pig, although the reconstructed embryos fused by electrical stimulation could be simply activated and subsequently developed to the blastocyst stage (Wang *et al.*, 1998; Cheong *et al.*, 2002), the fused embryos can develop to blastocysts with high rate when they were exposed to an additional activation treatment (Cheong *et al.*, 2000; Koo *et al.*, 2000; Polejaeva *et al.*, 2000). These observations indicate that appropriate activation protocol is necessary to insure the high rate of development and enhance the quality of NT embryos. Parthenogenetic activation of oocytes is a useful tool to examine the effects of recipient oocyte maturational age and activation protocol on the development of NTs. The present study was conducted to examine the effects of maturational age of oocytes and activation protocol on the development of parthenogenetic embryos (parthenotes).

MATERIALS AND METHODS

In Vitro Maturation of Oocytes

Porcine ovaries were collected from a slaughterhouse and transported to the laboratory in normal saline at 25 to 30℃. Cumulus-oocyte-complexes (COCs) were collected by aspiration from antral follicles 3 to 6 mm in diameter with an 18 G needle and a 10 ml syringe. COCs were washed three times in maturation medium (see below) and about 50 to 100 COCs were cultured in a 500 μl drop of NCSU-23 medium (16) supplemented with 0.6 mM cysteine (Sigma, St. Louis, MO, USA), 10 IU/ml PMSG (Sigma), and 10 IU/ml hCG (Sigma), 10 ng/ml EGF (Sigma), 10% (v/v) porcine follicular, and 50 μg/ml gentamicin, covered with paraffin oil and incubated for 22 hr at 39℃ in a humidified atmosphere of 5% CO_2 in air. COCs were then cultured in maturation medium without hormone for 8 to 22 hr at 39℃ in an atmosphere of 5% CO_2 in air. After maturation, cumulus cells were removed by vortexing the COCs in the presence of 0.1% PVA (Sigma) and 0.1% hyaluronidase (Sigma).

Evaluation of Nuclear Maturation

Oocytes were fixed by whole-mount method at 30 to 44 hr every 2 hr interval, and stained with 1% aceto-orcein. Oocytes with first polar body and metaphase chromosomes were considered to be in the MII stage.

MPF Kinase Assay

MPF kinase assay was carried out by measuring the activity of its catalytic subunit, cdc2 kinase, with MESACUP cdc2 kinase assay kit (MBL, Nagoya, Japan), as described by Anas *et al.* (2000). The correlation coefficients between cdc2 kinase activity examined by the MESACUP cdc2 kinase assay kit and histone H1 kinase activity measured by the radioactive method were as high as 0.9961. All chemical reagents were purchased from the Sigma unless otherwise noted. Briefly, each twenty oocytes were denuded from their cumulus cells at 36 and 44 hr of IVM, and washed twice with the cdc2 kinase sample buffer containing 50 mM Tris HCl, 0.5 M NaCl, 5 mM EDTA, 2 mM EGTA, 0.01% Brij 35, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.05 mg/ml leupeptin, 50 mM 2-mercaptoethanol, 25 mM β -glycerophosphate and 1 mM Na-orthovanadate. Oocytes were then transferred to a microtube containing 5 μl of the buffer and stored frozen at −80℃. At the time of assay, oocytes were lysed by successive freezing and thawing with liquid nitrogen and water. Five microliters of oocyte extract were mixed with 45 μl kinase assay buffer containing 25 mM Hepes buffer (MBL), 10 mM $MgCl_2$ (MBL), 10% biotinylated MV peptide (Ser-Lue-Tyr-Ser-Ser-Ser-Pro-Gly-Gly-Ala-Tyr-Cys; MBL) and 0.1 mM ATP, and were incubated for 30 min at 30℃. The phosphorylation reaction was terminated with 200 μl of stop reagent (PBS containing 50 mM EDTA; MBL), and centrifuged for 15 sec at 13,000 × g. For the detection of cdc2 kinase by ELISA, each 100 μl of the reaction mixture was transferred to each microwell strip coated with monoclonal antibody recognizing the phosphorylated form of the biotinylated MV peptide. The microwells were incubated at 25℃ for 60 min, and then washed 5 times with washing solution (PBS). One hundred microliters of horseradish peroxidase conjugated streptavidin solution were added to each well and then incubated at 25℃ for 30 min. After washing 100 μl of the POD substrate solution were added, and incubated for additional 5 min. Finally 100 μl of stop solution (20% H_3PO_4) was added to each well, and the optical density of each well was read at 492 nm with a microplate reader.

Parthenogenetic Activation

The MII oocytes were placed between two 0.2 mm diameter wire electrodes 1-mm apart, overlaid with 0.3 M mannitol solution containing 0.1 mM $MgSO_4$, 0.5 mM $CaCl_2$, and 0.05 mg/ml BSA. Activation was induced with a single DC pulse of 1.5 kV/cm for 30 μsec (ES) by an Electro Cell Manipulator 200 (BTX, San Diago, CA). Some oocytes were further activated 1 hr after the initial activation treatment by exposure to two DC pulses of 1.0 kV/cm for 50 μsec (ES+ES). After electrical activation treatment, some oocytes were treated with 2 mM 6-dimethlyaminopurine (DMAP) in PZM-3 supplemented with 3 mg/ml BSA for 4

hr before *in vitro* culture (EF+ES+D).

In Vitro Culture

After activation treatment, parthenotes were cultured in 50 μl droplets of PZM-3 supplemented with 3 mg/ml BSA overlaid with paraffin oil for 6 days under an atmosphere of 5% CO_2 in humidified air at 39℃. At days 2 and 6 after culture, the cleavage and development to the blastocyst stage were examined, respectively. The cell numbers in blastocysts were counted by staining with Hoechst 33342.

Statistical Analysis

At least three replicate trials for each treatment were performed. Percentage data, the activity of cdc2 kinase and cell number were analyzed by Duncan's multiple range tests using the General Linear Models procedure in the Statistical Analysis System (SAS Institute, Inc., Cary, NC).

RESULTS

Maturation Patterns of Porcine Oocytes

Oocytes were matured by 10.9% after 30 hr of maturation culture, and the maturation rate significantly increased as culture period was increased to 36 hr (66.9%, $p<0.05$). Thereafter, the rate was gradually increased, and finally reached 87.1% at 44 hr of culture (Fig. 1).

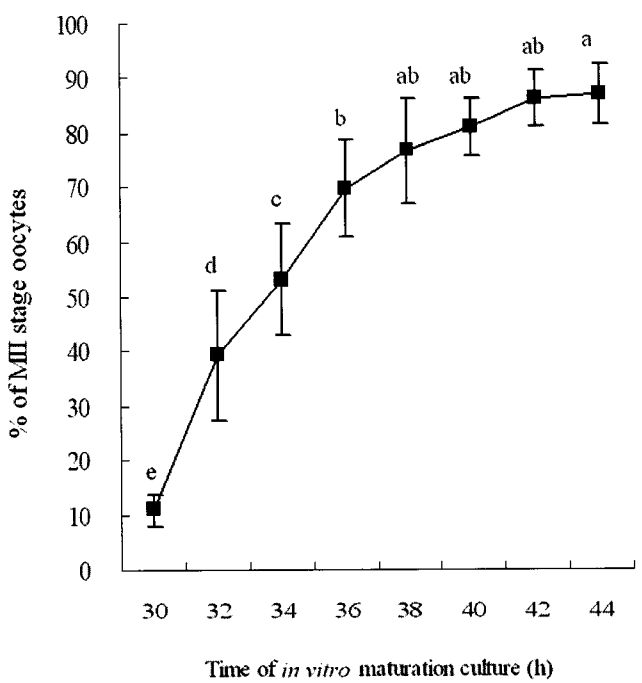

Fig. 1. Nuclear maturation rate of porcine oocytes cultured *in vitro*. Metaphase-II stage was examined at the times indicated. The data was presented as means±SE from four replicate trials. [a~e] Values with different letters are significantly different ($p<0.05$).

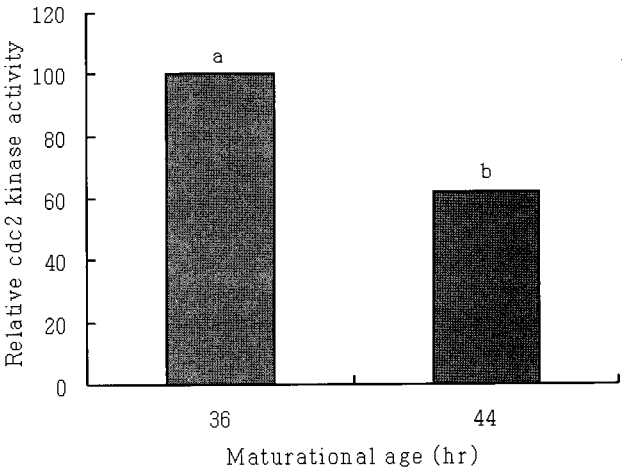

Fig. 2. The activity of cdc2 kinase of porcine oocytes matured *in vitro*. The level of the activity in oocytes from 36 hr of maturational culture is defined as 100%. Data are expressed as relative percentage of the level of $p34^{cdc2}$ activity in porcine oocytes. [a,b] Bars with different letters are significantly different ($p<0.05$).

cdc2 Kinase Activity

The cdc2 kinase activity was significantly decreased ($p<0.05$) with prolonged maturational culture period from 36 hr to 44 hr (Fig. 2).

Development of Parthenotes

Lower blastocyst formation rate (4.3%, $p<0.05$) were obtained by ES in 36 hr-matured oocytes compared to other treatments (16.5 and 20.5%) in the same age and the same treatment in 44 hr-matured oocytes (15.0%). Blastocyst formation rate (23.6%, $p<0.05$) was high by ES+ES+D in 44 hr-matured oocytes compared to ES (15.0%) and ES+ES (17.8%) treatment groups (Table 1). Whereas, there was no difference in the blastocyst formation rate by ES+ES+D between parthenotes activated at 36 and 44 hr of IVM (20.5 and 23.6 %, respectively).

DISCUSSION

The maturation timing of cultured porcine oocytes is different between individual oocytes (Ye *et al.*, 2002). The oocytes matured rapidly may undergo the various aging process, such as decreasing maturation promoting factor (MPF) activity (Kikuchi *et al.*, 1995) and unknown factors involved in oocyte quality in their cytoplasm than those matured lately. In the present study, although the matured oocytes were ob-

Table 1. Effects of maturational age and activation condition on development of porcine parthenotes

Maturational age (hr)	Activation condition*	No. of oocytes	No. (%) of embryos		Cell no. in blastocysts (Mean±SE)
			2-Cell	Blastocyst	
36	ES	161	62(38.5)[a]	7(4.3)[a]	22.0±1.2
	ES+ES	158	72(45.6)[ab]	26(16.5)[b]	30.6±3.7
	ES+ES+D	166	101(60.8)[b]	34(20.5)[bc]	25.3±2.2
44	ES	160	84(52.5)[ab]	24(15.0)[b]	29.0±3.1
	ES+ES	163	84(51.5)[ab]	29(17.8)[b]	27.9±2.7
	ES+ES+D	161	82(50.9)[ab]	38(23.6)[c]	25.5±2.4

* ES, electrical stimulation (one pulse of 1.5 kV/cm for 30 μsec.); ES+ES, ES followed by electrical stimulation (two pulses of 1.0 kV/cm for 50 μsec.) 1 hr apart; ES+ES+D, combination of ES+ES and DMAP culture for 4 hr.

[a~c] Values with different superscripts in the same column are significantly different ($p<0.05$).

served at 30 hr of maturation culture, the matured oocytes from 36 hr of culture were used as a newly matured recipient cytoplast to obtain the sufficient oocytes for this experiment. Whereas, matured oocytes at 44 hr were served as controls. In previous study, it was reported that the MPF activity levels from 38 hr to 48 hr of maturation culture were not different (Kagii *et al.*, 2000, Wehrend *et al.*, 2001). However, in this experiment, the MPF activity of MII oocytes cultured for 36 hr was significantly higher than that of MII oocytes cultured for 44 hr. It may be due to the differences in the experimental conditions, such as the culture conditions of oocyte maturation (Naito *et al.*, 1992; Ito *et al.*, 2001).

Oocytes can be activated by artificial stimulation that induces a rise in the intracellular concentration of calcium, but a single calcium increase can not induce the full activation of oocytes (Susko-Parrish *et al.*, 1994; Soloy *et al.*, 1997), because young oocytes continuously synthesize new cytostatic factor, which preserves MPF and maintains the meiotic arrest (Fissore and Robl, 1992; Yang *et al.*, 1994).

In the present study, some oocytes activated at 44 hr of IVM were developed to the blastocyst stage without additional activation stimulus. Whereas, blastocyst development were significantly restricted when oocytes were activated at 36 hr of IVM without additional activation stimulus, which may be due to differences in activation susceptibility between maturational ages of oocytes (Hagen *et al.*, 1991; Cheong *et al.*, 2000). Thus an additional activation treatment is necessary to induce the sufficient activation of oocytes matured for 36 hr (Cheong *et al.*, 2000; Verma *et al.*, 2000). It was suggested that a combined treatment is able to induce the full activation of oocytes through preventing reactivation of MPF (Liu *et al.*, 1998). The development rates to the blastocyst stage did not differ between 36 hr- and 44 hr-matured oocytes when they were activated with a combination of electrical stimulation and DMAP.

In conclusion, the result indicates that the developmental efficiency of porcine parthenotes depends on the interactions of oocyte maturational age and activation condition, and that additional activation is necessary to induce the activation of 36 hr-matured young oocytes. A treatment with the combination of electrical stimulus and DMAP can enhance the blastocyst formation rates of porcine parthenotes derived from both 36 hr- and 44 hr-matured oocytes.

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