Activation by Combined Treatment with Cycloheximide and Electrical Stimulation of *In-Vitro* Matured Porcine Oocytes Improves Subsequent Parthenogenetic Development

Kenji Naruse, Hong Rye Kim, Young Min Shin, Suk Min Chang, Hye Ran Lee, Vaishali Tarte, Yan Shi Quan, Beak Chul Kim, Tae Young Park, Su Min Choi, Chang Sik Park and Dong Il Jin†

Research Center for Transgenic and Cloned Pigs, Chungnam National University, Daejeon 338-708, Korea

ABSTRACT

Electrical treatment has been widely used for porcine oocytes activation. However, developmental rates following electrical activation of porcine oocytes is relatively inefficient compared to other domestic animals. To investigate the effects of porcine oocytes on combined activation by both chemical and electrical treatment, *in-vitro* matured oocytes were activated by combined cycloheximide and electrical pulses treatment. Cumulus-free oocytes were exposed with NCSU-23 medium containing cycloheximide (10 μg/ml) for 0, 5, 10, 20, 30 min and then activated by electrical pulse treatment and cultured in PZM-3 for 8 days. Also effects of exposure to 6.25 μM calcium ionophore for 2 min for cumulus-free oocytes were tested. The percentage of blastocyst formation in 10 min exposure to 10 μg/ml cycloheximide and electrical pulse treatment was significantly increased ($P<0.05$) than in the control group. And exposure to 6.25 μM calcium ionophore for 2 min with 10 μg/ml cycloheximide for 10min and electrical pulse treatment significantly increased ($P<0.05$) the percentage of blastocyst developmental rates than the control group. In conclusion, activation by combined cycloheximide and electrical stimulation treatment promoted the subsequent development of porcine oocytes and improved the subsequence blastocyst development.

(Key words : Porcine oocytes; Cycloheximide; Electrical stimulation; Parthenogenetic development; *In vitro* culture)

INTRODUCTION

Electrical activation and/or chemical activation have been used in oocytes of most species for the parthenogenetic induction. Mammalian embryos can be artificially induced to undergo parthenogenesis *in vitro* by a two-step protocol involving electrical pulses and/or treatment with a chemical agent (ionophore, ethanol or ionomycin) to elevate Ca^{2+} levels, followed by application of protein phosphorylation (6-dimethylaminopurine) or an inhibitor of protein synthesis (cycloheximide).

Since the first successful production of cloned pigs from cultured fetal fibroblast cells was reported using electrical activation (Polejaeva *et al.*, 2000), activation protocol for porcine somatic cell nuclear transfer have been fixed as electric stimulation. However, survival rates of somatic cell nuclear transferred porcine embryos were very low (Im *et al.*, 2003). At best, only about 20~30% of pig embryos develop to blastocysts in culture following parthenogenetic activation (Zhu *et al.*, 2002; Iwamoto *et al.*, 2005). And porcine developmental rates following activation is relatively inefficient compared to other domestic animals. Chemical activation for porcine oocytes proved to be detrimental while electrical activation seems to be a more suitable method than chemically activated (Yamauchi *et al.*, 1996).

Beneficial effects of cycloheximide treatment have been reported in matured mouse oocytes (Sirard *et al.*, 1978; Clarke and Marui, 1983) and in bovine oocytes (Sirard *et al.*, 1989). Although many studies on porcine oocytes activation have been demonstrated (Jilek *et al.*, 2000; Yi and Park, 2005), the activation by combined treatment with both cycloheximide and electrical stimulation of *in vitro* matured porcine oocytes have not been previously reported. Therefore, the objective of this study was to investigate the combined treatment of the matured oocytes with cycloheximide and electrical activation on subsequent developmental capacity of porcine oocytes *in vitro*.

MATERIALS AND METHODS

Oocyte Collection and *In Vitro* Maturation

Ovaries were collected from prepubertal gilts at a local slaughter house and transported to laboratory in 0.9% NaCl solution containing 100 IU/ml penicillin and 50 μg

* This work was supported by grants No R11-2002-100-03001-0 the ERC program of the Korean Science & Engineering Foundation and by a grant (Code # 20050301034440) from BioGreen 21 program, Rural Development Administration, Korea.

† Corresponding author : Phone: +82-42-821-5876, E-mail: dijin@cnu.ac.kr

/ml streptomycin at 30~35℃, then stored in a water bath at 37℃ before use. Cumulus-oocyte complexes (COCs) were aspirated from ovarian follicles 2~6 mm in diameter using 10-ml syringe fixed with an 18-gauge needle. COCs were washed three times in TL-Hepes containing 0.1% (w/v) polyvinyl alcohol (PVA). The oocytes was matured in bovine serum albumin (BSA)-Free NCSU-23 (Petters and Wells, 1993) supplemented with 0.57 mM L-cysteine, 10% (v/v) porcine follicular fluid, 2% (v/v) Basal Medium Eagle amino acids (BEM amino acid solutions, B-6766, Sigma), 1% (v/v) Minimum Essential Medium non-essential amino acids (MEM amino acid solutions, M-7145, Sigma), 0.5 μg /ml LH (L-5269, Sigma), 0.5 μg/ml FSH (F-2292, Sigma), 10 ng/ml epidermal growth factor (E-4127, Sigma), 100 U/ml penicillin, 5 μg/ml streptomycin. The oocytes were transferred into 500 μl of maturation medium which had been covered with mineral oil in a 4-well multidish (Nunc, Roskide, Denmark) and incubated for 42~44 h at 38.5℃ in an atmosphere of 5% CO_2 in air with maximum humidity. After 22 h of maturation culture, the oocytes were washed three times and transferred into 500 μl of basic medium without hormone for additional 22 h of culture. Most of the reagents used in this study were purchased from Sigma Chemical Corp (St Louis, MO, USA).

Activation and *In Vitro* Culture

Cumulus cells were removed from the oocytes by pipetting in TL-Hepes supplemented with 0.1% PVA and 0.1% hyaluronidase. The oocytes were transferred to activation solution consisting of 0.3 M D-mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 0.01% PVA, and washed three times. Cumulus-free oocytes were activated by a direct current pulse of 1.5 kV/cm for a duration of 100 μsec using an BTX Elector-Cell Manipulator 2001 (BTX, San Diego, CA, USA). PZM-3 containing 0.3% BSA (Yoshida *et al.*, 2002) was used for culture medium. After stimulation, oocytes were washed and transferred into 500 μl of culture media covered with mineral oil in a 4-well multidish. The oocytes were then cultured in groups of 500 μl of culture medium under 38.5℃ in an atmosphere of 5% CO_2 in air for 8 days.

Evaluation of Developmental Ability of Parthenogenetically Activated Oocytes

Activated oocytes were subjected to *in vitro* culture for 8 days. At 48~72 h after activation, cleavage of oocytes was evaluated under stereo microscope and blastocysts formation was examined on day 8. Day 7 blastocysts were stained with Hoechst 33342 (2 mg/ml in 2.3% sodium citrate) and counted at fluorescent microscope (Olympus, Japan).

Statistical Analysis

At least four replicate were conducted for each experiment. The significant difference among treatment groups was determined by ANOVA analysis. A P value less than 0.05 denoted a statistically significant difference.

Experimental Designs

At least four replicate were conducted for each experiment. In experiment 1, the effect of activation by combined cycloheximide and electrical pulses on development of porcine activated oocytes in PZM-3 was investigated. The matured oocytes were exposed with NCSU-23 medium containing cycloheximide (10 μg/ml) for 0, 5, 10, 20 and 30 min and then activated by electrical pulses treatment and activated oocytes were cultured in PZM-3 for 8 days (Fig. 1). In experiment 2, cumulus-free oocytes were exposed without or with 6.25 μM calcium ionophore for 2 min and were cultured in PZM-3 for 8 days.

RESULTS

Maturation rate of matured oocytes obtained after IVM was 85.7 % and parthenogenetically activated porcine

Table 1. Effects of combined activations by cycloheximide and electrical pulse by different exposure time of cycloheximide on *in vitro* development of porcine oocytes

Length of exposure (min)	No. of oocytes	No. of oocytes cleaved (Mean%±SE)	No. of blastocysts (Mean%±SE)	Cell number (Mean±SE)
0	167	148(88.6±5.0)	30(18.0±0.3)^{b}	31.2±5.4
5	169	148(87.6±5.0)	33(19.5±0.7)^{ab}	33.2±3.8
10	173	155(89.6±6.2)	45(26.0±1.2)^{a}	32.6±3.4
20	174	159(91.4±6.2)	34(19.5±1.3)^{ab}	30.6±2.6
30	180	145(80.6±5.7)	32(17.8±2.5)^{b}	30.2±6.6

^{a,b} Values with different superscripts are significantly different (P<0.05).

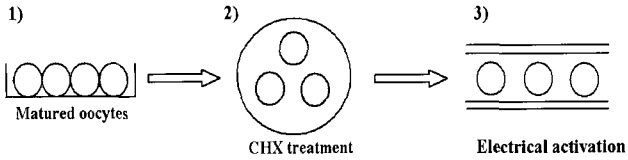

Fig. 1. Method for different parthenogenetic activation treatment in porcine oocytes. (1) *In vitro* maturation for 44hours, (2) Chemical activation (0, 5, 10, 20 and 30 min exposure to 10 μg/ml cycloheximide), (3) Electric activation (1.5 kV/cm, 100 μsec) and then *in vitro* culture in PZM-3 for 8 days.

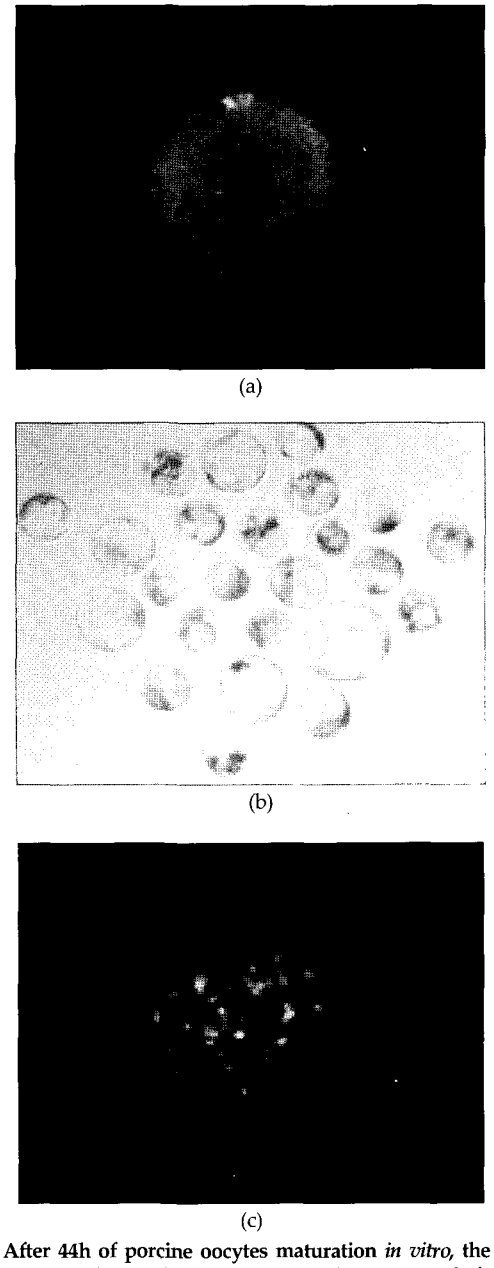

Fig. 2. After 44h of porcine oocytes maturation *in vitro*, the oocytes were stained with Hoechst 33258. Matured oocytes with first polar body and metaphase II plate (A), Parthenogenetic blastocysts (B), Nuclei of parthenogenetic blastocysts were stained with Hoechst (C).

oocytes were cultured in PZM-3 under 5% CO_2 and 20% O_2 in this experiment. Day 7 blastocysts were produced by activation with one 100 μsec pulse of 1.5 kV/cm DC. After 44 h, the oocytes at Metaphase II stage were stained with Hoechst 33258. And nuclei numbers of parthenogenetic blastocysts were stained with Hoechst 33342 and observed on fluorescent microscope (Fig. 2).

Although there was no significant difference in cleavage rates and total cell number of blastocysts between cycloheximide-treated groups and the control group, 10 min exposure to 10 μg/ml cycloheximide and electrical pulse treatment significantly increased ($P<0.05$) the percentage of blastocyst developmental rates than the control group (Table 1). Exposure to 10 μg/ml cycloheximide for 30 min followed by incubation significantly decreased ($P<0.05$) the percentage of blastocyst developmental rates than the exposure to 10 μg/ml cycloheximide for 10 min. Exposure to 6.25 μM calcium ionophore for 2 min with 10 μg/ml cycloheximide for 10 min and electrical pulse treatment increased also the percentage of blastocyst developmental rates than the control group (Table 2). However, electrical activation only decreased blastocyst developmental rate compared to cycloheximide-treated group or calcium ionophore-treated group with cycloheximide ($P< 0.05$).

DISCUSSION

Activation of oocytes matured *in vitro* is essential for the success of animals cloning by nuclear transfer (NT). Oocytes activation such as ionophore, ethanol and electrical pulses has been widely used for the artificial activation of mammalian embryos. Parthenogenetic activation of oocytes can be induced in several species by chemical activation, including calcium ionophore (Ware *et al.*, 1989), strontium (Marcus, 1990), ethanol (Presicce and Tang, 1994a; Presicce and Tang, 1994b), cycloheximide (Presicce and Tang, 1994a; Presicce and Tang, 1994b), and 6-dimethylaminopurine (Bodart *et al.*, 1999) and electrical pulses (Yamauchi *et al.*, 1996; Iwamoto *et al.*, 2005). Cycloheximide was shown to induce oocytes activation in mouse and in cattle (Sirard *et al.*, 1978; Clarke and Marui, 1983; Sirard *et al.*, 1989). Also, the oocytes activated with combined ethanol and cycloheximide treatment were high for both young and aging oocytes (Presicce and Tang, 1994a; Presicce and Tang, 1994b). The activation by combined treatment of *in vitro* matured porcine oocytes with cycloheximide, cytochalasin B and 6-dimethylaminopurine significantly increased the cleavage and blastocyst formation rates (Bodart *et al.*, 1999). The combined treatment of *in vitro* matured oocytes with cycloheximide and electrical activation improved parthenogenetic development of bovine oocytes (Yang *et al.*,

Table 2. Parthenogenetic development of porcine oocytes following electric pulse treatment or cycloheximide treatment with electric pulse or calcium ionophore plus cycloheximide with electric pulse treatment

Treatments	No[4]	No. of oocytes cleaved (Mean%±SE)	No. of blastocysts (Mean%±SE)
Electric activation[1]	155	132(85.2±3.5)	29(18.7±0.7)[b]
CHX[2] + Electric activation	159	139(87.4±2.2)	44(27.7±0.5)[a]
Ionophore[3] + CHX[2] + Electric activation	160	143(89.4±3.7)	49(30.6±1.2)[a]

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

[1] One DC pulse of 1.5 kV/cm for100 sec, [2] Cycloheximide (10 μg/ml) for 10 min,

[3] Ionophore (6.25 μM) for 2 min, [4] Number of porcine oocytes cultured in 4 replicate experiments.

1992). The combined treatment of *in-vitro* matured porcine oocytes with cycloheximide and ionophore significantly increased the activation rates and the developmental rates following parthenogenetic activation (Sirard *et al.*, 1989). And porcine oocytes meiotically arrested by CHX (cycloheximide) before maturation not only retain but also improve their developmental competence (Ye *et al.*, 2005). In generally, chemical activated oocytes with cycloheximide [CHX] or 6-dimethylaminopurine [6-DMAP] for 1~4 hr has been shown to enhance the activation rates and the parthenogenetic development in rabbits, cattle and goats (Liu *et al.*, 2002; Galli *et al.*, 2002; Lan *et al.*, 2005). Electric fusion and chemical activated oocytes with 6-DMAP enhanced development and higher cell number, while the combined treatment of porcine NT oocytes with electric fusion, cycloheximide did not enhance the *in vitro* development (Kim *et al.*, 2005). Recently oocyte activation is important for improving development of nuclear recipients for the effective production of cloned pigs. Our results suggest that the activation by combined treatment of *in vitro* matured porcine oocytes with cycloheximide plus electrical stimulation significantly increased the blastocyst formation rates. While manipulated bovine oocytes were carried out by comparing the effects of a 3~4 h two different chemical activation treatments (ionomycin/ cycloheximide [CHX] vs. iomomysin/6-dimethylaminopurine [6-DMAP]) (Galli *et al.*, 2002), shorter exposure of porcine oocytes to cycloheximide (10 min) and electrical activation significantly increased the activation and blastocyst developmental rates. And in our study, chemical agent (calcium ionophore) to elevate Ca^{2+} levels, followed by application of an inhibitor of protein synthesis (cycloheximide) and electrical pulse treatment did also effect the development of porcine oocytes *in vitro*. Thus, the treatment with electric pulse combined with protein synthesis inhibitor (cycloheximide) is effective method to high rates of parthenogenetic blastocyst development *in vitro* matured porcine oocytes.

REFERENCES

1. Bodart JF, Bechard D, Bertout M, Gannon J, Rousseau A, Vilain JP, Flament S (1995): Activation of Xenopus eggs by the kinase inhibitor 6-DMAP suggests a differential regulation of cyclin B and p39(mos) proteolysis. Exp Cell Res 253:413-421.
2. Clarke HJ, Marui Y (1983): The induction of reversible and irreversible chromosome decondensation by protein synthesis inhibition of meiotic maturation of mouse oocytes. Dev Biol 97:291-301.
3. Galli C, Lagutina I, Vassiliev I, Duchi R, Lazzari G (2002): Comparison of microinjection (piezo-electric) and cell fusion for nuclear transfer success with different cell types in cattle. Cloning Stem Cells 4:189-196.
4. Jilek F, Huttelova R, Petr J, Holubova M, Rozinek J (2000): Activation of pig oocytes using calcium ionophore: effect of protein synthesis inhibitor cycloheximide. Anim Reprod Sci 63:101-111.
5. Im GS, Liangxue L, Zhonghua L, Yanhong H, David W, Aaron B, Randall SP (2003): *In vitro* development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. Theriogenology 61:1125-1135.
6. Iwamoto M, Onishi A, Fuchimoto D, Somfai T, Takeda K, Tagami T, Hanada H, Noguchi J, Kaneko H, Nagai T, Kikuchi K (2005): Low oxygen tension during *in vitro* maturation of porcine follicular oocytes improves parthenogenetic activation and subsequent development to the blastocyst stage. Theriogenology 63:1277-1289.
7. Kim YS, Lee SL, Ock SA, Balasubramanian S, Choe SY, Rho GJ (2005): Development of cloned pig embryos by nuclear transfer following different activation treatments. Mol Reprod Dev 70:308-313.
8. Lan GC, Han D, Wu YG, Han ZB, Ma SF, Liu XY, Chang CL, Tan JH (2005): Effects of duration, concentration, and timing of ionomycin and 6-dimethylaminopurine (6-DMAP) treatment on activation of goat oocytes. Mol Reprod 71:380-388.
9. Liu CT, Chen CH, Cheng SP, Ju JC (2002): Parthenogenesis of rabbit oocytes activated by different stimuli. Anim Reprod Sci 70:267-276.

10. Marcus GJ (1990): Activation of cumulus-free mouse oocytes. Mol Repred Dev 26:159-162.
11. Petters RM, Wells KD (1993): Culture of pig embryos. J Reprod Fertil 48:61-73.
12. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH (2000): Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407:505-509.
13. Presicce GA, Tang X (1994a): Nuclear dynamics of parthenogenetic development of bovine oocytes matured *in vitro* for 20 and 40h and activated by combined by ethanol and cycloheximide treatment. Mol Reprod 37:61-68.
14. Presicce GA, Tang X (1994b): Parthenogenetic development of bovine oocytes matured *in vitro* for 24h and activated by ethanol and cycloheximide. Mol Reprod 38:380-385.
15. Sirard MA, Whittingham DG, Molinaro M, Vivarelli E (1978): Parthenogenetic activation of mouse oocytes induced by inhibitors of protein synthesis. J Embryol Exp Morphol 43:157-166.
16. Sirard MA, Florman HM, Leibfried-Rutledge ML, Barnes FL, First NL (1989): Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biol Reprod 40:1257-1263.
17. Ware CB, Barnes FL, Maiki-Laurila M, First NL (1989): Age dependence of bovine oocytes activation. Gamete Res 22:265-275.
18. Yamauchi N, Sasada H, Sugawara S, Nagai T (1996): Effect of culture conditions on artificial activation of porcine oocytes matured *in vitro*. Reprod Fertil 8:1153-1156.
19. Yang X, Jiang S, Shi Z (1992): Improved activation by combined cycloheximide and electrical pulse treatment of bovine follicular oocytes matured *in vitro* for 23~24h. Biol Reprod (Suppl.) 46:117 (abstract).
20. Ye J, Campbell KHS, Craigon J, Luck MR (2005): Dynamic changes in meiotic progression and improvement of developmental competence of pig oocytes *in vitro* by follicle stimulating hormone and cycloheximide. Biol Reprod 72:399- 406.
21. Yi YJ, Park CS (2005): Parthenogenetic development of porcine oocytes treated by ethanol, cycloheximide, cytochalasin B and 6-dimethylaminopurine. Anim Reprod Sci 86:297-304.
22. Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S (2002): Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol Reprod 66:112-119.
23. Zhu J, Telfer EE, Fletcher J, Springbett A, Dobrinsky JR, De Sousa PA, Wilmut I (2002): Improvement of an electrical activation protocol for porcine oocytes. Biol Reprod 66:635-641.

(Received: 2 Jan. 2006/Accepted: 6 Mar. 2006)