Effect of Kinetin on *In Vitro* Development of Parthenogenetic Porcine Oocytes Exposed to Demecolcine Prior to Activation

Kiyoung Kim, Sang Kyu Park and Sangho Roh[*]

Laboratory of Embryo Biotechnology and Cellular Reprogramming, Dental Research Institute and BK21 CLS, Seoul National University School of Dentistry, Seoul 110-749, Republic of Korea

ABSTRACT

This study was designed to investigate the effect of kinetin on *in vitro* development of parthenogenetic porcine oocytes exposed to demecolcine prior to activation. *In vitro* matured metaphase II stage oocytes were incubated in 0 or 2 μg/ml demecolcine supplemented defined culture medium for 3 h and the oocytes were activated electrically. The parthenogenetic porcine embryos were then cultured in 0 or 200 μM kinetin supplemented defined culture medium for 7 days. Regardless of demecolcine treatment, kinetin supplementation increased blastocyst rates significantly (7.0% versus 12.1% and 4.9% versus 8.5%; Control versus Kinetin and Demecolcine versus Kinetin + Demecolcine, respectively, $p<0.05$). Demecolcine treatment before activation tended to decrease blastocyst rates regardless of kinetin supplementation although it is not statistically significant. Total cell numbers in the blastocysts also tended to be elevated in embryos when supplemented with kinetin, however only the result between Kinetin and Demecolcine groups is statistically significant (37.6 ± 7.2 versus 28.1 ± 9.5, respectively, $p<0.05$). In conclusion, the present report shows that kinetin enhances developmental competence of parthenogenetic porcine embryo regardless of demecolcine pre-treatment before parthenogenetic activation when they were developed in defined culture condition.

(Key words : kinetin, demecolcine, porcine embryo, *in vitro* culture, parthenogenesis)

INTRODUCTION

In the previous study, we reported that kinetin enhances *in vitro* development of porcine somatic cell nuclear transfer (SCNT) embryos and also improves total cell number of parthenogenetic and SCNT blastocysts (Won *et al.*, 2008). Kinetin is one of the N[6]-substituted adenine derivates, which are part of the larger family of plant hormones (Miller *et al.*, 1955, 1956). Kinetin promotes cell division by abbreviating the cell cycle (Bayliss, 1985; Francis and Sorrell, 2001) and is likely generated as a secondary oxidative damage product of DNA (Barciszewski *et al.*, 1997). Furthermore, the presence of kinetin bases in DNA stimulates the expression of repair enzymes, superoxide dismutase activity, and ribosomal RNA transcription (Barciszewski *et al.*, 1997; Olsen *et al.*, 1999). Although our group recently reported positive effects of kinetin on SCNT porcine embryo development *in vitro* (Won *et al.*, 2008), it is still not clear that which step of SCNT procedure was affected by kinetin supplementation.

Demecolcine also known as colcemid is a drug being used in chemotherapy. It disrupts microtubules then inhibits cell division causing mitotic block (Rieder and Palazzo, 1992). It is also reported that demecolcine induces loss of sex chromosomes in *Drosophila melanogaster* (Rodriguez-Arnaiz *et al.*, 2004). Demecolcine-derived enucleation technique is recently used for porcine SCNT (Yin *et al.*, 2002b). Since demecolcine treatment in metaphase II oocytes induces visible cytoplasmic extrusion including chromosomes and may be able to minimize the loss of mRNA and ooplasmic factors during enucleation process, this enucleation technique is being widely applied to SCNT in rabbits (Yin *et al.*, 2002a), mice (Gasparrini *et al.*, 2003) and cattle (Tani *et al.*, 2006) as well as in pigs (Yin *et al.*, 2002b).

In the previous report, we found that kinetin only increases *in vitro* development of SCNT porcine embryos to the blastocyst stage not the parthenogenetic counterpart although it improves total cell number in parthenogenetic blastocysts as well as SCNT ones (Won *et al.*, 2008). The different processes between SCNT

and parthenogenesis in our protocol are demecolcine-derived enucleation and somatic cell injection resulting cell reprogramming. As first step, the present study reports the effect of kinetin on *in vitro* development of parthenogenetic porcine oocytes exposed to demecolcine prior to electrical activation.

MATERIALS AND METHODS

1. Chemicals

All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) unless otherwise stated.

2. Oocyte Recovery and *In Vitro* Maturation (IVM)

Slaughterhouse ovaries were collected from 5 to 6-month-old prepubertal gilts (100 ± 10 kg of body-weight), placed in saline at $30 \sim 35\,^{\circ}\text{C}$, and transported within 2 h to the laboratory. After washing with saline three times, cumulus-oocyte complexes (COCs) were recovered by aspiration of 2- to 5-mm follicles using an 18-gauge hypodermic needle attached to a 5-ml disposable syringe. After washing three times in IVM medium, COCs that were enclosed by more than three layers of compact cumulus cells and an evenly granulated ooplasm were selected for IVM. Selected COCs were cultured in 4-well culture dishes (Nunc, Denmark) containing 500 μl of IVM medium under warmed and gas-equilibrated mineral oil for $46 \sim 48$ h at $38.5\,^{\circ}\text{C}$ and 5% CO_2. The IVM medium for oocytes is composed of tissue culture medium 199 with Earle's salts and L-glutamine (TCM199, Gibco Life Technologies Inc., USA) supplemented with 26.2 mM $NaHCO_3$, 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 10 ng/ml epidermal growth factor, 10 IU/ml equine chorionic gonadotropin and human chorionic gonadotropin, and 0.1% (v/v) polyvinylalcohol (PVA).

3. Demecolcine Treatment

The COCs were denuded for 1 min by vortexing and were incubated in North Carolina State University-23 medium (NCSU-23) supplemented with 2 μg/ml demecolcine and 1 mg/ml PVA for 3 h at $38.5\,^{\circ}\text{C}$ and 5% CO_2. The oocytes in control group were treated the same except demecolcine treatment.

4. Parthenogenesis and *In Vitro* Culture

Electrical activation was performed at room temperature using a CF-150/B electro-cell fusion system (BLS, Hungary) in a chamber that contained two stainless steel electrodes that were 1.0 mm apart and that was filled with activation buffer. Oocytes were activated with a 1.6 kV/cm DC pulse for 40 μsec in 0.26 M mannitol supplemented with 0.1 mM $MgSO_4$, 0.05 M $CaCl_2$, and 0.01% PVA. The activated oocytes were treated for 5 to 6 h in NCSU-23 supplemented with 5 μg/ml cytochalasin B, 1 mg/ml PVA and either 0 or 200 μM kinetin. The oocytes were then cultured in 20-μl drops ($10 \sim 15$ oocytes per drop) of NCSU-23 with 1 mg/ml PVA and either 0 or 200 μM kinetin for 7 days at $38.5\,^{\circ}\text{C}$ and 5% CO_2 after rinsing with appropriate culture medium nine times. Detailed experimental design was described in Fig. 1.

5. Statistic analysis

Experiments were repeated five times, and mean values were analyzed by ANOVA analysis of variance. Difference at $p < 0.05$ was considered significant.

RESULTS

Regardless of demecolcine treatment, kinetin supplementation increased blastocyst rates significantly (7.0% versus 12.1%

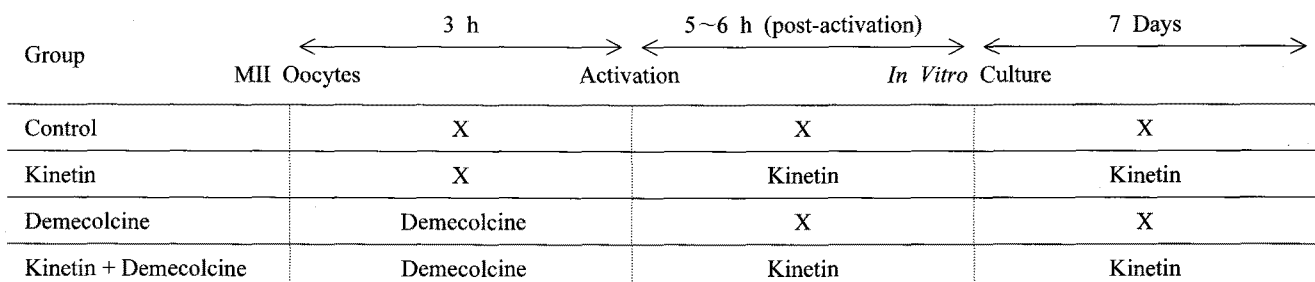| Group	3 h	5~6 h (post-activation)	7 Days
	MII Oocytes → Activation	→ *In Vitro* Culture	→
Control	X	X	X
Kinetin	X	Kinetin	Kinetin
Demecolcine	Demecolcine	X	X
Kinetin + Demecolcine	Demecolcine	Kinetin	Kinetin

Fig. 1. Schematic explanation of kinetin supplementation of demecolcine pre-treated parthenogenetic porcine oocytes. Bidirectional arrows indicate the period of kinetin and/or demecolcine exposure to the oocytes in culture before or after activation. Kinetin was supplemented to the activated oocytes during post-activation (to prevent haploidization) and *in vitro* culture period. Demecolcine was treated to the matured oocytes prior to electrical activation.

and 4.9% versus 8.5%; Control versus Kinetin and Demecolcine versus Kinetin + Demecolcine, respectively, $p<0.05$, Table 1). Demecolcine treatment before activation tended to decrease blastocyst rates regardless of kinetin supplementation although it is not statistically significant. Total cell numbers in the blastocysts also tended to be elevated in embryos when supplemented with kinetin, however only the result between Kinetin and Demecolcine groups is statistically significant ($p<0.05$, Table 1).

DISCUSSION

The result above indicates that positive effect of kinetin on the development of parthenogenetic porcine embryos *in vitro* regardless of demecolcine pre-treatment before activation. In our previous report, although kinetin enhanced porcine embryo development derived both from parthenogenesis and SCNT (Won *et al.*, 2008), the significant effect of kinetin on the development to the blastocyst stage was limited to the SCNT group whereas blastocyst expansion and cell number of embryos were improved by kinetin supplementation both in parthenogenesis and SCNT. There are two remarkable differences between parthenogenesis and SCNT procedures in the previous report (Won *et al.*, 2008). The first one is of course somatic cell injection process following enucleation resulting in cell reprogramming. More beneficial effect of kinetin in SCNT than parthenogenesis may be from the improvement of repair pathway through receptor-mediated signaling by kinetin (Rattan and Clark, 1994) or preventing DNA damage resulting from oxidative stress in SCNT porcine embryos as the presence of kinetin bases in DNA stimulates the expression of repair enzymes and superoxide dismutase activity (Barciszewski *et al.*,

1997) as we described previously (Won *et al.*, 2008). The second difference other than nuclear injection is demecolcine-derived enucleation. Demecolcine is a microtubule disrupter and has been used for chemotherapy (Rieder and Palazzo, 1992). Demecolcine treatment in porcine metaphase II stage oocytes induces visible cytoplasmic extrusion including chromosomes although the mechanisms of action of demecolcine are not clear (Yin *et al.*, 2002b) and this may be able to minimize the loss of mRNA and ooplasmic factors during mechanical enucleation. Although demecolcine-aided chromosome extrusion makes enucleation easier in porcine SCNT (Yin *et al.*, 2002b) and is also known that compartmentalization of chromatin is reversible (Gasparrini *et al.*, 2003), our data shows that demecolcine treatment before activation tended to be detrimental for *in vitro* development of parthenogenetic porcine embryos.

Interestingly, the present study shows that kinetin increased the blastocyst formation rate significantly regardless of demecolcine treatment and this is different from the previous report (Won *et al.*, 2008) which only showed increased blastocyst expansion/hatching and total cell number in blastocysts. This difference is may be from different activation timing or other experimental conditions. In the experiment here, parthenogenetic activation was delayed 3 h when compared with in the previous experiment (Won *et al.*, 2008) because the oocytes here were exposed to the culture medium with or without demecolcine for 3 h to mimic our SCNT protocol. Delayed activation or extended exposure of matured oocytes to *in vitro* culture medium for 3 h before activation may affect developmental competence of parthenogenetic porcine oocytes. Kinetin may be more potent when aged oocytes are used for parthenogenesis or SCNT in the pig. It is known that kinetin inhibits activation of phospholipase C and leads to lower intracellular

Table 1. Effect of kinetin on *in vitro* development of parthenogenetic porcine embryos exposed to demecolcine prior to activation

Group[*]	Number of activated oocytes	Number of 2-cells (%)	Number of blastocysts (%)	Total cell number in blastocysts (mean ± SD)
Control	288	180 (62.7)	20 (7.0)^{ab}	32.4 ± 3.8^{ab}
Kinetin	303	215 (70.9)	38 (12.1)^{c}	37.6 ± 7.2^{a}
Demecolcine	287	164 (56.6)	12 (4.9)^{b}	28.1 ± 9.5^{b}
Kinetin + Demecolcine	295	179 (61.3)	25 (8.5)^{ac}	32.3 ± 7.4^{ab}

Five replicates.
[*]See Fig. 1 for detailed experimental design and determination of group names.
Values with different superscripts (a~c) in the same column significantly differ ($p<0.05$).

Ca^{2+} oscillations (Sheu et al., 2003). In the present study, kinetin might enhance developmental competence of aged oocytes after delayed activation by preventing excessive intracellular Ca^{2+} concentrations caused by Ca^{2+} oscillations in the endocytoplasmic reticulum and could control intracellular Ca^{2+} concentration during the activation event.

In conclusion, we have shown that kinetin enhances developmental competence of parthenogenetic porcine embryo regardless of demecolcine pre-treatment before parthenogenetic activation when they the embryos were developed in defined culture condition.

REFERENCES

Barciszewski J, Siboska GE, Pedersen BO, Clark BF and Rattan SI. 1997. A mechanism for the in vivo formation of N^6-furfuryladenine, kinetin, as a secondary oxidative damage product of DNA. FEBS Lett. 414:457-460.

Bayliss M. 1985. Control of cell division in cultured cells. In: Bryant JA, Francis D, editors. The Cell Division Cycle in Plants., Cambridge University Press, Cambridge, pp. 157-178.

Francis D and Sorrell DA. 2001. The interface between the cell cycle and plant growth regulators: A mini review. Plant Growth Regul. 33:1-12.

Gasparrini B, Gao S, Ainslie A, Fletcher J, McGarry M, Ritchie WA, Springbett AJ, Overstrom EW, Wilmut I and De Sousa PA. 2003. Cloned mice derived from embryonic stem cell karyoplasts and activated cytoplasts prepared by induced enucleation. Biol. Reprod. 68:1259-1266.

Miller CO, Skoog F, Okumura FS, von Saltza MH and Strong FM. 1956. Isolation, structure and synthesis of kinetin, a substance promoting cell division. J. Am. Chem. Soc. 78: 1375-1380.

Miller CO, Skoog F, von Saltza MH and Strong FM. 1955. Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc. 77:1329-1334.

Olsen A, Siboska GE, Clark BF and Rattan SI. 1999. N(6)-Furfuryladenine, kinetin, protects against Fenton reaction-mediated oxidative damage to DNA. Biochem. Biophys. Res. Commun. 265:499-502.

Rattan SI and Clark BF. 1994. Kinetin delays the onset of ageing characteristics in human fibroblasts. Biochem. Biophys. Res. Commun. 201:665-672.

Rieder CL and Palazzo RE. 1992. Colcemid and the mitotic cycle. J. Cell. Sci. 102(3):387-392.

Rodriguez-Arnaiz R, Sortibran AC and Tellez GO. 2004. Detection of mitotic recombination and sex chromosome loss induced by adriamycin, chlorambucil, demecolcine, paclitaxel and vinblastine in somatic cells of Drosophila melanogaster in vivo. Mutagenesis 19:121-127.

Sheu JR, Hsiao G, Shen MY, Chou CY, Lin CH, Chen TF and Chou DS. 2003. Inhibitory mechanisms of kinetin, a plant growth-promoting hormone, in platelet aggregation. Platelets. 14:189-196.

Tani T, Shimada H, Kato Y and Tsunoda Y. 2006. Demecolcine-assisted enucleation for bovine cloning. Cloning Stem Cells 8:61-66.

Won C, Park SK, Cho SG, Min BM and Roh S. 2008. Kinetin enhances in vitro development of parthenogenetic and nuclear transfer porcine embryos. Mol. Reprod. Dev. 75:1701-1709.

Yin XJ, Kato Y and Tsunoda Y. 2002a. Effect of enucleation procedures and maturation conditions on the development of nuclear-transferred rabbit oocytes receiving male fibroblast cells. Reproduction 124:41-47.

Yin XJ, Tani T, Yonemura I, Kawakami M, Miyamoto K, Hasegawa R, Kato Y and Tsunoda Y. 2002b. Production of cloned pigs from adult somatic cells by chemically assisted removal of maternal chromosomes. Biol. Reprod. 67:442-446.