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

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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 N4-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 \textit{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 \textit{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 30−35°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 46−48 h at 38.5°C and 5% CO₂. 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 NaHCO₃, 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 10 mg/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°C and 5% CO₂. The oocytes in control group were treated the same except demecolcine treatment.

4. Parthenogenesis and \textit{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 MgSO₄, 0.05 M CaCl₂, 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 (10−15 oocytes per drop) of NCSU-23 with 1 mg/ml PVA and either 0 or 200 μM kinetin for 7 days at 38.5°C and 5% CO₂ after rinsing with appropriate culture medium nine times. Detailed experimental design was described in Fig. 1.

5. Statistical 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%)

<table>
<thead>
<tr>
<th>Group</th>
<th>MII Oocytes</th>
<th>3 h Activation</th>
<th>5−6 h (post-activation)</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Kinetin</td>
<td>X</td>
<td>Kinetin</td>
<td>Kinetin</td>
<td>Kinnet</td>
</tr>
<tr>
<td>Demecolcine</td>
<td>Demecolcine</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Kinetin + Demecolcine</td>
<td>Demecolcine</td>
<td>Kinnet</td>
<td>Kinnet</td>
<td></td>
</tr>
</tbody>
</table>

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 \textit{in vitro} culture period. Demecolcine was treated to the matured oocytes prior to electrical activation.
Effect of Kinetin on the Development of Demecolcine Pre-treated Oocytes

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 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

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of activated oocytes</th>
<th>Number of 2-cells (%)</th>
<th>Number of blastocysts (%)</th>
<th>Total cell number in blastocysts (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>288</td>
<td>180 (62.7)</td>
<td>20 (7.0)bc</td>
<td>32.4 ± 3.8ab</td>
</tr>
<tr>
<td>Kinetin</td>
<td>303</td>
<td>215 (70.9)</td>
<td>38 (12.1)c</td>
<td>37.6 ± 7.2a</td>
</tr>
<tr>
<td>Demecolcine</td>
<td>287</td>
<td>164 (56.6)</td>
<td>12 (4.9)e</td>
<td>28.1 ± 9.5b</td>
</tr>
<tr>
<td>Kinetin + Demecolcine</td>
<td>295</td>
<td>179 (61.3)</td>
<td>25 (8.5)e</td>
<td>32.3 ± 7.4ab</td>
</tr>
</tbody>
</table>

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 endocytic 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


