Effects of Cycloheximide on Parthenogenetic Development of Pig Oocytes Activated by Ultrasound Treatment

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Abstract. The present study was carried out to examine the parthenogenetic development of pig oocytes treated with different concentrations of cycloheximide for different durations following activation by ultrasound stimulation. When oocytes were treated with 10 µg/ml cycloheximide for 5 h was significantly (P<0.05) higher than those of oocytes treated for 0–2 h. The blastocyst formation rate of oocytes treated with 0–10 or 50 µg/ml cycloheximide for the same duration. When oocytes were treated with different concentrations of cycloheximide for 2 h, however, the blastocyst formation rate of oocytes treated with 40 µg/ml cycloheximide was significantly (P<0.05) higher than those of oocytes treated with 0–10 or 50 µg/ml cycloheximide. The blastocyst formation rate of oocytes treated with 10 µg/ml cycloheximide for 5 h was significantly different from that of oocytes treated with 40 µg/ml cycloheximide for 2 h. These treatments did not affect the activation status of oocytes compared with controls that were not treated with cycloheximide. The results of the present study showed that cycloheximide improves the parthenogenetic development of pig oocytes activated by ultrasound stimulation.

Key words: Activation, Cycloheximide, Parthenogenetic development, Pig, Ultrasound

Successful development of cloned embryos produced using nuclear transfer depends on artificial stimulation-induced activation [1]. Electric stimulation is the most common method of activation of embryos in somatic cell nuclear transfer studies that have succeeded in producing cloned piglets [2–11]. Recently, we have shown that ultrasound stimulation can induce nuclear activation and parthenogenetic development of pig oocytes matured in vitro [12]. Ultrasound stimulation is also useful for inducing activation and in vitro development of cloned embryos derived from miniature pig somatic cells [13]. In addition, miniature pig cloned embryos activated by ultrasound stimulation have the ability to develop into piglets after transfer to recipient females [14]. The advantage of the activation protocol using ultrasound is that it can treat more oocytes and embryos at one time compared with the activation protocol using electric pulses [12]. This advantage is valuable in somatic cell nuclear transfer studies requiring activation of a lot of cloned embryos. Moreover, ultrasound is considered to be better artificial stimulation for activation of embryos than electric pulses in production of cloned miniature pigs because no piglets have been obtained from embryos activated by electric pulses [14]. Therefore, it would be useful to optimize the activation protocol that uses ultrasound.

Combined treatments of electric stimulation with cycloheximide, a nonspecific protein synthesis inhibitor, enhances the activation and in vitro development of pig oocytes and cloned embryos derived from somatic cells [15–19]. These reports suggest that an additional treatment with cycloheximide is necessary to improve the developmental capacity of pig oocytes and cloned embryos activated by ultrasound. In the present study, therefore, we determined whether cycloheximide treatment improves parthenogenetic development of pig oocytes after activation by ultrasound stimulation.

Materials and Methods

In vitro maturation of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in saline at 32–36 C. The follicular contents were recovered by aspiration from follicles (2–5 mm in diameter) using an 18-gauge needle (Terumo, Tokyo, Japan) and a 5-ml disposable syringe (Nipro, Osaka, Japan). The cumulus-oocyte complexes (COCs) were gathered from the follicular contents and washed twice with HEPES (Nacalai Tesque, Kyoto, Japan)-buffered Tyrode-lactate-pyruvate-polyvinyl alcohol (PVA; Sigma-Aldrich Chemical, St. Louis, MO, USA) and a 5-ml droplet of the maturation medium, respectively. Only COCs possessing a compact cumulus mass and evenly granulated ooplasm were selected. COCs in groups of 40–50 were transferred to a droplet of the maturation medium (200 µl) under paraffin oil (Nacalai Tesque) in a 35-mm polystyrene dish (Becton Dickinson, Franklin Lakes, NJ, USA) and cultured at 38.5 C in an atmosphere of 5% CO2 in air. The maturation medium consisted of 90% (v/v) TCM-199 with Earle’s salts (Gibco BRL, Grand Island, NY, USA) supplemented with 0.91 mM sodium pyruvate (Sigma), 3.05 mM D-glucose (Wako Pure Chemical, Osaka, Japan), 0.57 mM cysteine hydrochloride hydrate (Sigma), 10 ng/ml epidermal growth factor (Sigma), 10 IU/ml eCG (Teikoku-Zoki, Tokyo, Japan), 10 IU/ml HCG (Teikoku-Zoki), 100 µg/ml amikacin sulfate (Meiji Seika, Tokyo, Japan), 0.1% (w/v)
containing 100 μl of mounting medium consisting of glycerol and PBS (9:1) mixed and spun at 300 rpm during ultrasound exposure. After exposure, the oocytes were transferred into 50 μl of the same medium. The ultrasound probe (8 mm in diameter) of a KITAC-3000 Sonopore (Rich Mar, Inola, OK, USA) was inserted directly into the activation medium, and the oocytes were exposed to 2872-KHz ultrasound at an intensity of 45 V for 30 sec with a 10 Hz burst rate and 30% duty cycle. A miniature stirrer was placed within the well and spun at 300 rpm during ultrasound exposure. After exposure, the oocytes were transferred into 50 μl of mPZM-3 supplemented with different concentrations of cycloheximide (Nacalai Tesque) and incubated for different durations under 5% CO2, 5% O2 and 90% N2 at 38.5 C. During the first 2 h of the incubation, 2.2 μg/ml cytochalasin B was added to each medium to prevent extrusion of a second polar body. After each treatment, the oocytes were transferred into 50 μl of mPZM-3 and culture was continued. At 2 days of culture, the oocytes were examined for cleavage. The oocytes were assessed for blastocyst formation at 7 and 8 days (experiments 1 and 4) or 8 days (experiments 2 and 3) of culture. At the end of culture, the blastocysts were mounted, fixed for 72 h in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein acid in 45% (v/v) acetic acid and examined for polar body extrusion and pronuclear formation under a Nomarski differential interference microscope (Olympus, Tokyo, Japan). Oocytes with a pronucleus were regarded as activated oocytes.

Table 1. Parthenogenetic development of pig oocytes exposed to ultrasound and treated with 10 μg/ml cycloheximide for different durations

<table>
<thead>
<tr>
<th>Duration of treatment (h)</th>
<th>No. of oocytes cultured</th>
<th>No. (%)b of oocytes developed to 2-cell (Day 2)c</th>
<th>Mean no. of blastocysts SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>110</td>
<td>38 (34.5)d</td>
<td>64.9 ± 8.8</td>
</tr>
<tr>
<td>2</td>
<td>109</td>
<td>56 (51.4)d</td>
<td>54.6 ± 4.0</td>
</tr>
<tr>
<td>5</td>
<td>108</td>
<td>80 (74.1)d</td>
<td>52.3 ± 2.9</td>
</tr>
<tr>
<td>10</td>
<td>109</td>
<td>90 (82.6)d</td>
<td>50.0 ± 3.1</td>
</tr>
</tbody>
</table>

Experiments were repeated six times. Percentage per oocytes cultured. The time of examination (Day 0= oocyte activation).

Values with different superscripts within each column are significantly different (at least P<0.05).

PVA and 10% (w/v) pig follicular fluid. After 40–46 h of culture, cumulus cells were removed by pipetting with 0.1% (w/v) hyaluronidase (Sigma). Oocytes with a polar body were selected for the experiments.

Activation and culture of oocytes

In vitro-matured and denuded oocytes in groups of 50–70 were washed twice in activation medium composed of 250.3 mM sorbitol, 0.5 mM Ca(CH3COO)2, 0.5 mM Mg(CH3COO)2 and 0.1% (w/v) BSA [20] and then transferred to a well of a 4-well plate (Nunc, Roskilde, Denmark) containing 800 μl of the same medium. The ultrasound probe (8 mm in diameter) of a KITAC-3000 Sonopore (Rich Mar, Inola, OK, USA) was inserted directly into the activation medium, and the oocytes were exposed to 2872-KHz ultrasound at an intensity of 45 V for 30 sec with a 10 Hz burst rate and 30% duty cycle. A miniature stirrer was placed within the well and spun at 300 rpm during ultrasound exposure. After exposure, the oocytes were transferred into 50 μl of mPZM-3 and culture was continued. At 2 days of culture, the oocytes were examined for cleavage. The oocytes were assessed for blastocyst formation at 7 and 8 days (experiments 1 and 4) or 8 days (experiments 2 and 3) of culture. At the end of culture, the blastocysts were placed on slides with a drop of mounting medium consisting of glycerol and PBS (9:1) containing 100 μg/ml Hoechst 33342 (Sigma). A cover slip was placed on top of the blastocysts, and the edge was sealed with nail polish. The number of nuclei was counted under ultraviolet light.

Experimental designs

In experiment 1, the parthenogenetic development of oocytes treated with cycloheximide for different durations was examined. After exposure to ultrasound, oocytes were treated with 10 μg/ml cycloheximide for 0, 2, 5 or 10 h.

In experiment 2, the parthenogenetic development of oocytes treated with different concentrations of cycloheximide for 5 h was examined. Oocytes were treated with 0, 5, 10, 15 or 20 μg/ml cycloheximide.

In experiment 3, the parthenogenetic development of oocytes treated with different concentrations of cycloheximide for 2 h was examined. Oocytes were treated with 0, 10, 20, 30, 40 or 50 μg/ml cycloheximide.

In experiment 4, the parthenogenetic development of oocytes treated with 10 μg/ml cycloheximide for 5 h was compared with that of oocytes treated with 40 μg/ml cycloheximide for 2 h.

In experiment 5, the activation status of oocytes treated with 10 μg/ml cycloheximide for 5 h was compared with that of oocytes treated with 40 μg/ml cycloheximide for 2 h. At 10–12 h of culture, the oocytes were mounted, fixed for 72 h in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein acid in 45% (v/v) acetic acid and examined for polar body extrusion and pronuclear formation under a Nomarski differential interference microscope (Olympus, Tokyo, Japan). Oocytes with a pronucleus were regarded as activated oocytes.

Statistical analysis

All percentage data were subjected to an arcsin transformation in each replicate. The transformed values and numbers of cells in blastocysts were analyzed by one-way ANOVA followed by Fisher’s protected least significant difference test. A probability of P<0.05 was considered statistically significant.

Results

Experiment 1

When oocytes were treated with cycloheximide for 5–10 h, the cleavage rates (74.1–82.6%) were significantly (P<0.05) higher than those (34.5–51.4%) of oocytes treated for 0–2 h (Table 1). There were no significant differences in the blastocyst formation rates (5.7–13.0%) of oocytes among the different durations of treatment when they were observed at 7 days of culture. However, the blastocyst formation rates (21.1–26.9%) of oocytes treated for 5–10 h were significantly (P<0.05) higher than that (8.2%) of oocytes treated for 0 h at 8 days of culture. In addition, the blastocyst formation rate (26.9%) of oocytes treated for 5 h was significantly (P<0.05) higher than that (12.8%) of oocytes treated for 2 h. The mean numbers of cells (50.0–64.9 cells) in the blastocysts were not affected by the different durations of treatment.
Experiment 2
There were no significant differences in the rates of oocytes cleaved (51.9–77.6%) among the different concentrations of cycloheximide (Table 2). However, the blastocyst formation rate (43.9%) of oocytes treated with 10 μg/ml cycloheximide was significantly (P<0.05) higher than those (22.1–27.1%) of oocytes treated with 0–5 or 15–20 μg/ml cycloheximide. The mean numbers of cells (52.0–55.4 cells) in the blastocysts were not affected by the different concentrations of cycloheximide.

Experiment 3
The cleavage rates (60.7–71.2%) of oocytes were not affected by the different concentrations of cycloheximide (Table 3). In contrast, the blastocyst formation rates (29.1–36.4%) of oocytes treated with 20–40 μg/ml cycloheximide were significantly (P<0.05) higher than those (15.4–17.3%) of oocytes treated with 0 or 50 μg/ml cycloheximide. Moreover, the blastocyst formation rate (36.4%) of oocytes treated with 40 μg/ml cycloheximide was significantly (P<0.05) higher than that (22.9%) of oocytes treated with 10 μg/ml cycloheximide. There were no significant differences in the mean numbers of cells (61.5–67.8 cells) in the blastocysts among the different concentrations of cycloheximide.

Experiment 4
There were no significant differences in the rates of oocytes cleaved (42.5–62.9%) among the different cycloheximide treat-

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**Table 2.** Parthenogenetic development of pig oocytes exposed to ultrasound and treated with different concentrations of cycloheximide for 5 h

<table>
<thead>
<tr>
<th>Concentration of cycloheximide (μg/ml)</th>
<th>No. of oocytes cultured</th>
<th>No. (%)(^b) of oocytes developed to</th>
<th>Mean no. ± SEM of cells in blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥2-Cell (Day 2)(^c)</td>
<td>Blastocyst (Day 8)(^c)</td>
</tr>
<tr>
<td>0</td>
<td>104</td>
<td>54 (51.9)</td>
<td>23 (22.1)(^d)</td>
</tr>
<tr>
<td>5</td>
<td>107</td>
<td>73 (68.2)</td>
<td>29 (27.1)(^d)</td>
</tr>
<tr>
<td>10</td>
<td>107</td>
<td>83 (77.6)</td>
<td>47 (43.9)(^e)</td>
</tr>
<tr>
<td>15</td>
<td>104</td>
<td>72 (69.2)</td>
<td>27 (26.0)(^d)</td>
</tr>
<tr>
<td>20</td>
<td>105</td>
<td>77 (73.3)</td>
<td>26 (24.8)(^d)</td>
</tr>
</tbody>
</table>

\(^a\)Experiments were repeated five times. \(^b\)Percentage per oocytes cultured. \(^c\)The time of examination (Day 0=oocyte activation). \(^d\)-\(^e\)Values with different superscripts are significantly different (at least P<0.05).

**Table 3.** Parthenogenetic development of pig oocytes exposed to ultrasound and treated with different concentrations of cycloheximide for 2 h

<table>
<thead>
<tr>
<th>Concentration of cycloheximide (μg/ml)</th>
<th>No. of oocytes cultured</th>
<th>No. (%)(^b) of oocytes developed to</th>
<th>Mean no. ± SEM of cells in blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥2-Cell (Day 2)(^c)</td>
<td>Blastocyst (Day 8)(^c)</td>
</tr>
<tr>
<td>0</td>
<td>117</td>
<td>71 (60.7)</td>
<td>18 (15.4)(^d)</td>
</tr>
<tr>
<td>10</td>
<td>118</td>
<td>76 (64.4)</td>
<td>27 (22.9)(^d)</td>
</tr>
<tr>
<td>20</td>
<td>117</td>
<td>75 (64.1)</td>
<td>34 (29.1)(^d)</td>
</tr>
<tr>
<td>30</td>
<td>118</td>
<td>84 (71.2)</td>
<td>36 (30.5)(^d)</td>
</tr>
<tr>
<td>40</td>
<td>118</td>
<td>82 (69.5)</td>
<td>43 (36.4)(^d)</td>
</tr>
<tr>
<td>50</td>
<td>110</td>
<td>71 (64.5)</td>
<td>19 (17.3)(^d)</td>
</tr>
</tbody>
</table>

\(^a\)Experiments were repeated five times. \(^b\)Percentage per oocytes cultured. \(^c\)The time of examination (Day 0=oocyte activation). \(^d\)-\(^f\)Values with different superscripts are significantly different (at least P<0.05).

**Table 4.** Parthenogenetic development of pig oocytes exposed to ultrasound and treated with or without cycloheximide

<table>
<thead>
<tr>
<th>Cycloheximide treatment</th>
<th>No. of oocytes cultured</th>
<th>No. (%)(^b) of oocytes developed to</th>
<th>Mean no. ± SEM of cells in blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥2-Cell (Day 2)(^f)</td>
<td>Blastocyst (Day 7)(^f)</td>
</tr>
<tr>
<td>134</td>
<td>132</td>
<td>57 (42.5)</td>
<td>16 (11.9)</td>
</tr>
<tr>
<td>10 μg/ml for 5 h</td>
<td>132</td>
<td>83 (62.9)</td>
<td>29 (22.0)</td>
</tr>
<tr>
<td>40 μg/ml for 2 h</td>
<td>133</td>
<td>79 (59.4)</td>
<td>21 (15.8)</td>
</tr>
</tbody>
</table>

\(^a\)Experiments were repeated seven times. \(^b\)Percentage per oocytes cultured. \(^c\)The time of examination (Day 0=oocyte activation). \(^d\)-\(^f\)Values with different superscripts are significantly different (P<0.01).
Ca²⁺ ionophore treatment [26], microinjection of Ca²⁺ into the cytoplasm of the oocyte leads to an increase in the intracellular Ca²⁺ concentration and may provide only a single intracellular Ca²⁺ transient of the oocyte [33]. Although electric stimulation leads to elevation of intracellular Ca²⁺ in the oocyte, it provides only a single Ca²⁺ transient instead of a Ca²⁺ oscillation [33]. A single Ca²⁺ transient can only relieve metaphase arrest in aging oocytes where protein synthetic capacity is diminished; however, in early developing oocytes, it may not be sufficient to induce complete inactivation of MPF [34]. This is believed to result in reactivation of MPF and mitogen-activated protein kinase [35] that prevents progress towards the first interphase.

A possible way to prevent the reactivation of MPF is to use protein synthesis or protein kinase inhibitors. Cycloheximide is an inhibitor of protein synthesis that can block the production of cyclin B, the regulatory component of MPF. Normally, cyclin B is produced during interphase of the cell cycle and is destroyed at the beginning of mitosis [36], and inhibiting its synthesis leads to a significant decrease in active MPF [37]. Treatment with cycloheximide has been used successfully to induce resumption of meiosis in mouse [38] and cattle [39] oocytes. In the pig, although incubation of oocytes in the presence of cycloheximide does not induce activation [15], cycloheximide enhances the activation and in vitro development of oocytes and cloned embryos derived from somatic cells induced by electric stimulation [15–19].

Ultrasound exposure is thought to induce the formation of pores in the cell membrane and permit easier inward transport of molecules or other agents into the cells across the membrane barrier, resulting in faster and more direct intracellular uptake [40, 41]. In addition, the concentration of Ca²⁺ in the medium used for exposure to ultrasound is an important factor for the activation status and parthenogenetic development of pig oocytes when they are activated by ultrasound [20]. Although the exact mechanism and process of oocyte activation by ultrasound are not fully understood, on the basis of these reports, it is suggested that pig oocytes are activated by an influx of extracellular Ca²⁺ via ultrasound-produced pores in the plasma membrane. Therefore, ultrasound stimulation may provide only a single intracellular Ca²⁺ transient of the oocyte and may not be sufficient to induce complete inactivation of MPF. Cycloheximide would prevent reactivation of MPF by blocking the production of cyclin B in pig oocytes after exposure to ultrasound and bring about improvements in their parthenogenetic development.

The blastocyst formation rate of the pig oocytes at 7 days of culture was not affected by the presence or absence of cycloheximide treatment after exposure to ultrasound. At 8 days of culture, however, improvement in the blastocyst formation rate by

### Table 5. Activation status of pig oocytes exposed to ultrasound and treated with or without cycloheximide

<table>
<thead>
<tr>
<th>Cycloheximide treatment</th>
<th>No. of oocytes examined</th>
<th>No. (%) of oocytes activated</th>
<th>No. (%) of activated oocytes with 1PB</th>
<th>2PB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1PN</td>
<td>2PN</td>
</tr>
<tr>
<td>10 μg/ml for 5 h</td>
<td>133</td>
<td>118 (88.7)</td>
<td>79</td>
<td>66.9</td>
</tr>
<tr>
<td>40 μg/ml for 2 h</td>
<td>135</td>
<td>116 (85.9)</td>
<td>77</td>
<td>66.4</td>
</tr>
</tbody>
</table>

*Experiments were repeated six times. *Percentage per oocytes examined. *Percentage per oocytes activated. *PB, polar body; PN, pronucleus.

The meiotic maturation of mammalian oocytes is spontaneously blocked at the metaphase II stage, and further progress of meiosis beyond the block depends on sperm-induced activation [21]. It is generally accepted that the metaphase promoting factor (MPF) is a key regulator of meiotic resumption [22, 23]. MPF is a heterodimer consisting of a catalytic subunit called cdc2 and a regulatory subunit called cyclin B [24]. During fertilization, the sperm induces oscillation in the intracellular Ca²⁺ concentration of the oocyte that leads to inactivation of MPF through Ca²⁺-dependent destruction of cyclin B [25]. Artificial stimulation, including Ca²⁺ ionophore treatment [26], microinjection of Ca²⁺ into the cytoplasm [27] and electric stimulation [28, 29], is able to induce parthenogenetic activation of *in vitro*-matured pig oocytes through elevation of the cytoplasmic Ca²⁺ concentration. Electric stimulation, the method most widely used to reinitiate the meiotic cell-cycle after nuclear transfer of somatic cells in the pig, results in formation of pores in the plasma membrane that facilitate the uptake of extracellular Ca²⁺ by the oocytes [30–32]. This influx of Ca²⁺ leads to an increase in the intracellular Ca²⁺ concentration and oocyte activation [33]. Although electric stimulation leads to elevation of intracellular Ca²⁺ in the oocyte, it provides only a single Ca²⁺ transient instead of a Ca²⁺ oscillation [33]. A single Ca²⁺ transient can only relieve metaphase arrest in aging oocytes where protein synthetic capacity is diminished; however, in early developing oocytes, it may not be sufficient to induce complete inactivation of MPF [34]. This is believed to result in reactivation of MPF and mitogen-activated protein kinase [35] that prevents progress towards the first interphase.

**Discussion**

The results of the present study show that cycloheximide improves the parthenogenetic development of pig oocytes activated by ultrasound stimulation and show that treatments with 10 μg/ml cycloheximide for 5 h and 40 μg/ml cycloheximide for 2 h are effective for their development *in vitro*. The blastocyst formation rates (11.9–22.0%) of oocytes at 7 days of culture were not affected by the different cycloheximide treatments. However, the blastocyst formation rates (30.1–34.8%) of oocytes treated with 10 μg/ml cycloheximide for 5 h or 40 μg/ml cycloheximide for 2 h were significantly (P<0.01) higher than that (13.4%) of oocytes treated without cycloheximide when they were observed at 8 days of culture. The mean numbers of cells (41.3–44.3 cells) in the blastocysts were not affected by the different cycloheximide treatments.

**Experiment 5**

The rates of activated oocytes (85.9–89.8%) were not affected by the different cycloheximide treatments (Table 5). There were no significant differences in the rates of activated oocytes without a second polar body (79.3–81.3%) among the different cycloheximide treatments.

The results of the present study show that cycloheximide improves the parthenogenetic development of pig oocytes activated by ultrasound stimulation and show that treatments with 10 μg/ml cycloheximide for 5 h and 40 μg/ml cycloheximide for 2 h are effective for their development *in vitro*.
cycloheximide was observed. Cycloheximide inhibits protein synthesis and thus blocks the development of pig oocytes activated by electric pulses at the pronuclear stage [42]. The development of pig oocytes exposed to ultrasound would be also inhibited by cycloheximide at the same stage, and restart of their development would take time after transfer into medium without cycloheximide. Therefore, their development probably begins later than that of non-treated oocytes, resulting in delayed blastocyst formation. Alternatively, pig oocytes treated with cycloheximide might develop slowly compared with non-treated oocytes.

In general, pig oocytes and cloned embryos are treated with 10 μg/ml cycloheximide for 5–6 h after application of electric pulses [16–19]. Although the same protocol was useful for improving the parthenogenetic development of pig oocytes exposed to ultrasound, treatment with 40 μg/ml cycloheximide for 2 h was also effective for improving their development. Naruse et al. [18] reported that pig blastocysts developed from somatic cell nuclear transferred embryos treated with 10 μg/ml cycloheximide for 6 h after electric activation contain significantly more apoptotic cells compared with controls that were not treated with cycloheximide. When the embryos were treated with 10 μg/ml cycloheximide for 10 min prior to electric activation, however, their blastocyst formation rate improved without increased apoptosis [18], although this protocol did not affect the parthenogenetic development of pig oocytes activated by ultrasound (unpublished data). It would be interesting to examine apoptosis in pig blastocysts developed from oocytes treated with 40 μg/ml cycloheximide for 2 h after activation by ultrasound stimulation. In any case, this protocol may be better than treatment with 10 μg/ml cycloheximide for 5 h in production of cloned piglets because cloned embryos can be transferred into recipient females quickly after activation.

In conclusion, we have established a novel and effective activation protocol for pig oocytes using a combined treatment of ultrasound exposure and cycloheximide. We expect that this protocol will be useful in activating cloned pig embryos produced by somatic cell nuclear transfer and bring about improvements in the efficiency of cloning.

Acknowledgments

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