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Effect of Activation Methods for Bovine Oocytes after Intracytoplasmic Injection

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Abstract. A principal nuclear transfer procedure is to inject a donor cell into the perivitelline space in an enucleated oocyte and then electric fusion is performed (cell fusion method). The effects of activation methods in reconstructed oocytes for the serum-starved somatic cell cloning procedure were investigated in this study by means of intracytoplasmic injection (ICI). Bovine oocytes were enucleated at 18–22 h for in vitro maturation, and subsequently the nucleus of cumulus cell collected from Japanese Black Bulls (JBCC) after 5–7 days of starved culture was injected into the recipient cytoplasm with a piezo-micromanipulator. At 1 h after ICI, reconstructed oocytes were stimulated with ethanol (ET) or calcium ionophore (CaI) as the first activation treatment, followed by cycloheximide (CHX) or 6-dimethylaminopurine (DMAP) treatment as the second activation. In the experiment on the first activation method, the proportion of reconstructed oocytes developing to the blastocyst stage was significantly ($p < 0.01$) higher in the ET activation method than that with CaI (10.5% and 4.7%, respectively). And the experiment on the second activation method after ET treatment showed similar proportions of blastocyst development in both CHX and DMAP treatments (5.9% and 2.8%, respectively). The present results indicated that combined activation treatment with ET and CHX was efficient for reconstructed bovine oocytes by ICI.

Key words: Intracytoplasmic injection, Activation treatment, Bovine

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After a report of the birth of “Dolly” in 1997 [1], somatic cell nuclear transfer techniques have developed remarkably. So far many offspring of sheep, cattle, goats, mice, pigs and other mammals have been obtained by nuclear transfer with various differentiated cells [2, 3], mammalian cells [1], muscle cells [4], fibroblast cells [5–8], cumulus cells [8–11] and other cells. The production of genetically identical mammals by somatic cell nuclear transfer, would bring many advantages in the acquisition of domestic animals with defined ability, selection of sex, improvement of productional efficiencies as to meat, milk and fur,

and improvement of efficiency in feeding and breeding. Furthermore, the production of medicines, and specific proteins in milk for medical application becomes possible with gene handling.

On somatic nuclear transfer, it was suggested that synchronization of the cell cycle was important for the recipient oocyte and donor cell [1, 12, 13]. DNA content in a recipient cytoplasm at metaphase II (MII) stage is 2C (C=amount of DNA). On the other hand, calcium-oscillation does not occur in reconstructed oocytes after nuclear transfer as in normally fertilized oocytes, so that reconstructed oocytes must be activated artificially for resumption of meiosis. A primary activation treatment with electric stimulus [14], ethanol (ET)

[4, 8, 15], ionomycin [7, 16] or calcium-ionophore (CaI) [4, 11, 17] induces a rise of Ca^{2+} concentration in oocytes, and with strontium [18, 19] induces calcium-oscillation. And as secondary activation treatment to support primary activation, cycloheximide (CHX) [4, 5, 20, 21], 6-dimethylaminopurine (DMAP) [16, 20], boheminine [21] and butyrolactone-I [7, 22] are generally used to culture reconstructed oocytes. But, there are great differences in the calcium wave between normally fertilized and parthenogenetic oocytes [6]. It has been suggested that these differences affect the developmental ability of reconstructed oocytes after activation. Furthermore, it has also been suggested that the cell fusion treatment induces the chimerization of mitochondria DNA in both donor cells and recipient oocytes. This has caused the problem of the authenticity of cloned oocytes.

In the present study, we used serum-starved cumulus cells from Japanese Black Bulls (JBCC) at G0/G1 phases (2C) as donor cells after measuring the DNA content by flow cytometry [5, 11, 23, 24, 25]. And the effects of methods for the activation of reconstructed oocytes by intracytoplasmic injection (ICI) with a piezo-micromanipulator were evaluated.

Materials and Methods

Oocyte collection and in vitro maturation

Ovaries were collected from Holstein cattle at a slaughterhouse and transported to the laboratory in physiological saline at 30–35 °C within 3 h, or after incubation for 20 h at 20 °C. Cumulus-oocyte complexes (COCs) were collected by aspirating follicles 2–8 mm in diameter with a 5 mL syringe and 18-gauge needle. The COCs were then cultured for 18–22 h in 100- μL drops (10–15 oocytes/drop) of Medium 199 (M-199 with Earle's salts, L-glutamine, 2,200 mg/L sodium bicarbonate and 25 mM HEPES buffer; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (v/v) heat-treated newborn calf serum (NBCS), 1 mM L-glutamine, 1 $\mu\text{g}/\text{mL}$ estradiol-17 β (Sigma, USA) and 0.02 AU/mL pFSH (Antrin; Denka Pharmaceutical Co., Ltd, Kawasaki, Japan), at 39 °C in 5% CO_2 and 95% humidified air. After *in vitro* maturation (IVM) for 18–22 h, the COCs were used for recipient oocytes.

Oocyte enucleation and staining

After IVM for 18–22 h, expanded cumulus cells were removed by pipetting and vortexing the COCs for 3–5 min in the presence of 0.1% (w/v) hyaluronidase. Denuded oocytes were washed 2–3 times in Hepes-buffered synthetic oviductal fluid (H-SOF) supplemented with 0.3% (w/v) bovine serum albumin (BSA; Sigma) and enucleated by removing the first polar body and the metaphase II plate with a small amount (approximately 10–30% of total volume) of surrounding cytoplasm in a 100 μL droplet of H-SOF supplemented with 2 $\mu\text{g}/\text{mL}$ cytochalasin B (Sigma) and 0.3% (w/v) BSA. After enucleation, the oocytes were washed 3–5 times in bicarbonate-buffered SOF (B-SOF) supplemented with 0.8% (w/v) BSA.

Chromosome removal was confirmed by Hoechst 33342 (Sigma) labeling under ultraviolet light (wavelength 360–380 nm). Each enucleated oocyte was placed in a 5 μL droplet of 0.5 $\mu\text{g}/\text{mL}$ Hoechst 33342, stained for 15–30 min and examined under a fluorescent microscope (Nikon). After staining, only successfully enucleated oocytes were immediately washed 3–5 times in B-SOF and placed in the same medium until intracytoplasmic injection (ICI).

Donor cell isolation

Primary cell lines were established from JBCC collected by aspirating as described previously. The collected cumulus cells were cultured in α minimal essential medium (α -MEM; Gibco-BRL) supplemented with 10% (v/v) NBCS. After reaching 70–100% confluence, the cells were cultured for ICI in α -MEM supplemented 0.5% (v/v) NBCS (serum starvation culture), or they were frozen in α -MEM in LN_2 after serum starvation for 5–7 days. Donor cells were trypsinized (0.25% (w/v) trypsin and 0.1% (w/v) EDTA in Dulbecco's PBS (–)(D-PBS(–)); Gibco-BRL) at 39 °C for 3–5 min at 15–30 min before ICI. The suspension was washed with D-PBS (–) supplemented with 5.6 mM glucose, 0.33 mM sodium pyruvate, 25 $\mu\text{g}/\text{mL}$ kanamycin and 1% (w/v) polyvinylpyrrolidone (PVP), and centrifuged at $300 \times g$ for 5 min.

Intracytoplasmic injection (ICI)

Donor cells were transferred to H-SOF containing 10% (w/v) PVP. Cells of small modal diameter (5–7 μm , probably G0/G1 phases) were selected and the nucleus was picked out from their

respective cells with a piezo-micromanipulator. Some nuclei were aspirated in the injection pipette, and the pipette containing each nucleus with a little cytoplasm was placed into a droplet of H-SOF and immediately injected directly into the cytoplasm of an enucleated oocyte by means of a piezo-micromanipulator.

Oocyte activation and culture

After injection for 1 h, nuclear transfer oocytes were activated with 7% ET or 5 μ M CaI for 5 min as the primary activation treatment. After 15 min, reconstructed oocytes were treated with 10 μ g/mL cycloheximide (CHX; Sigma) or 1.9 mM 6-dimethylaminopurin (DMAP; Sigma) and 2 μ g/mL cytochalasin B in B-SOF for 6 h as the secondary activation treatment. After the activation treatments, reconstructed oocytes were transferred into 30 μ L droplets of B-SOF, and incubated at 39 C in 5% CO₂, 5% O₂, 90% N₂ in humidified air for 8 days.

Flow cytometry & cell cycle analysis

Cumulus cells from Japanese Black Bulls (JBCC) were cultured in α -MEM supplemented with 10% (v/v) NBCS. The suspension of cells, which were cultured in α -MEM supplemented with 10% NBCS (normal culture) for 2 days or α -MEM supplemented with 0.5% NBCS for 5 days (serum starvation) after normal culture for 1–2 days, were washed twice in PBS(–). Addition of 1.1 mL PBS(–) was made to the cells, and 100 μ L of the cell suspension was used for cell counts and 1 mL cell suspension was fixed by slowly adding 2.3 mL ethanol (–20 C, 99.5%). After fixation, the samples were kept at –20 C until measurement of the cell cycle. Just before flow cytometry the samples were washed twice in PBS(–) and treated with 0.25 mg/mL RNase A (Sigma) for 30 min. After RNase A treatment, they were stained with 50 μ g/mL propidium iodide (PI; Sigma) for 30 min.

The DNA content of JBCC after normal culture for 2 days, or serum starvation for 5 days followed by a normal culture for 1–2 days, was measured with a flow cytometer (EPICS XL; Beckman Coulter., Tokyo, Japan). Seven to ten thousand cells were collected for the flow cytometry analysis with Mac Cycle software (Phoenix Flow Systems, Inc., San Diego, CA). Percentages of each phase at G0/G1, S and G2/M were calculated.

Experiment 1: Examination of activation methods after ICI

One hour after ICI, reconstructed oocytes were activated with 7% ET for 5 min followed by 10 μ g/mL CHX for 6 h (ET-CHX), 7% ET for 5 min alone (ET), or 10 μ g/mL CHX for 6 h alone (CHX). The results of the three activation methods were compared with the non-activation group.

Experiment 2: Examination of a primary activation method

One hour after ICI, reconstructed oocytes were activated with 7% ET for 5 min followed by 10 μ g/mL CHX for 6 h (ET-CHX) or 5 μ M CaI for 5 min and followed by 10 μ g/mL CHX for 6 h (CaI-CHX).

Experiment 3: Examination of a secondary activation method

One hour after ICI, reconstructed oocytes were activated with 7% ET for 5 min followed by 10 μ g/mL CHX (ET-CHX) or 1.9 mM DMAP (ET-DMAP) for 6 h.

Statistical analysis

The cell cycle study was repeated four times. Experiments 1–3 were repeated 4–5 times for blastocyst development of reconstructed oocytes. Percentages of all data for cell cycle phases and development of reconstructed oocytes were arcsine transformed and analyzed by the General Linear Models (GLM) Procedure with the Statistical

Table 1. Proportions of cell cycles in the donor cells treated with different serum concentrations

Serum conc. (%)*	G0/G1 phases (mean $\pm$ SEM: %)	S phase (mean $\pm$ SEM: %)	G2/M phases (mean $\pm$ SEM: %)
10	68.4 $\pm$ 1.9 ^b	20.4 $\pm$ 1.3 ^a	11.1 $\pm$ 1.9 ^a
0.5	90.2 $\pm$ 0.8 ^a	3.7 $\pm$ 0.4 ^b	5.9 $\pm$ 0.4 ^b

a-b; p<0.05.

*; Four replicates each.

Table 2. Activation methods for blastocyst development of reconstructed bovine oocytes

Activation method*	No. of oocytes cultured (n)	Blastocysts (mean $\pm$ SEM: %)
ET-CHX	60	11.7 $\pm$ 2.3 ^a
ET	58	1.7 $\pm$ 1.4 ^b
CHX	38	0 ^b
Non-treatment	34	0 ^b

a-b; $p < 0.01$.

*Four replicates each.

Table 3. Comparison of blastocyst development in primary activation substances

Activation substance*	No. of oocytes cultured (n)	Blastocysts (mean $\pm$ SEM: %)
ET-CHX	57	10.5 $\pm$ 0.9 ^a
CaI-CHX	64	4.7 $\pm$ 1.7 ^b

a-b; $p < 0.01$.

*Four replicates each.

Analysis System (SAS; Cary, USA). Treatment means were also compared with the Duncan's multiple range test.

Results

As a result of enucleation confirmation by fluorescence, the enucleation rate was $90.9 \pm 1.7\%$ ($n=591$), and no enucleation confirmation was performed during the experiments.

Proportions of the cell cycle in JBCC with the two serum concentrations are shown in Table 1. The proportion of cells at the G0/G1 phases with the serum starvation culture was significantly ($p < 0.05$) higher than that of normal-cultured cells ($90.2 \pm 0.8\%$ and $68.4 \pm 1.9\%$, respectively). We therefore used serum-starved cells in this study.

In Experiment 1, the reconstructed oocytes treated with both ET and CHX (ET-CHX) showed a significantly ($p < 0.01$) higher rate of blastocyst development than oocytes treated with ET (ET) or CHX (CHX) alone and the non-activation group (11.7%, 1.7%, 0% and 0%, respectively) (Table 2). In Experiment 2, on the effects of the primary activation method, rates of development to the blastocyst stage were significantly ($p < 0.01$) higher with the ET-CHX method than with the CaI-CHX method (10.5% and 4.7%, respectively) (Table 3). In Experiment 3, on the effects of the secondary

Table 4. Comparison of blastocyst development in secondary activation substances

Activation substance*	No. of oocytes cultured (n)	Blastocysts (%)
ET-CHX	34	5.9
ET-DMAP	36	2.8

*Five replicates each.

activation method, development rates to the blastocyst stage were 5.9% and 2.8% for the ET-CHX method and ET-DMAP method, respectively, and these figures are not significantly different (Table 4).

Discussion

The present results indicated that the proportion of cells at the G0/G1 phases by serum starvation was significantly higher than that in normal-cultured cells (Table 1), and the results have consisted with the report of Tani *et al.* [26]. As a result of artificial activation of the reconstructed oocytes, the proportion of blastocyst development was improved by both activation methods (primary and secondary), particularly with ET in the primary activation treatment and CHX in the secondary activation treatment.

Normal cell-cycle phases were composed of G1 (2C; DNA content), S (between 2C and 4C), G2 (4C) and M (4C). But it is difficult to obtain certain cells at a particular cell cycle stage, because in cell groups there were those of various cell-cycle phases. Therefore, cell-cycle phases need to be synchronized to G0/G1 by serum starvation [25], or by growing cultures to 100% confluence on the culture dish. It has been suggested that by using cells at G0/G1 phases as donor cells, it would be possible to avoid chromosomal abnormalities and DNA re-replication on reconstructed oocytes [1, 12, 27].

For nuclear transfer, two methods are commonly used. These are cell fusion and intracytoplasmic injection (ICI) methods [8, 28]. The former is used generally for bovine nuclear transfer, in which the donor cell is put into the perivitelline space between the zona pellucida and the cytoplasm of an enucleated oocyte, and then both cytoplasm are fused by electrical stimulation. In the latter, ICI was recently used for porcine and mice nuclear

transfer [15, 18, 19, 29, 30] to remove the cell membrane of the donor cell and inject only the nucleus into the cytoplasm of the recipient oocyte with a piezo-micromanipulator. Problems in the former method are the mixture of cytoplasm and mitochondria in both the donor cell and a recipient oocyte, and the authenticity of cloned oocytes by chimerization of mitochondria DNA. In this study we therefore used ICI with the piezo-micromanipulator for donor nucleus injection.

On the other hand, activation treatments for the functional maturation/meiosis/mitosis promoting factor (MPF) systems [31] are necessary for normal development of reconstructed oocytes. Because the MPF level in an M II oocyte as the recipient cell is high immediately after donor cell injection, nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) occur in the oocytes. Therefore, reconstructed oocytes were activated artificially after PCC to reduce activity of MPF for mitosis resumption. Livestock oocytes might not be activated sufficiently with only the primary activation treatment, and activation methods with the primary treatment together with the secondary treatment by CHX, DMAP or others are required to improve the development of reconstructed oocytes. It is suggested that the reascent of MPF activity is prevented by the secondary activation treatment [31]. In the present study, we obtained high developmental proportions (11.7%) of reconstructed oocytes by means of the secondary activation treatment. The secondary activation treatment was performed with cytochalasin B; cytokinesis (not nuclear division) inhibitor, to prevent physical injury and injury to factors participating in divisions after enucleation of M II oocytes, and to inhibit the release of a second polar body after resumption of second meiosis [32, 33]. The release of the second polar body from reconstructed oocyte reduces the number of chromosomes to half, and the oocytes would not develop normally.

In fertilized bovine oocytes, Ca^{2+} oscillation caused by sperm activation stimuli plays a part in inactivating MPF, and releases oocytes from metaphase arrest and allows the resumption of mitotic cycles. The maximum peak of the Ca^{2+} wave in Ca^{2+} oscillation by fertilization stimuli occurs immediately after fertilization, and thereafter the Ca^{2+} wave is observed for 3–4 h just before pronucleus formation. In contrast, the

electric stimulus as an artificial activation introduces a single Ca^{2+} wave [34]. And Ca^{2+} waves with ET and CaI differ from that of fertilization stimuli. It is suggested that these differences in Ca^{2+} oscillations might affect the proportions of blastocyst development after activation treatments. As an activation treatment for the resumption of mitosis in reconstructed oocytes, chemical substances, which cause a rise in the intracellular Ca^{2+} concentration or inhibitor of MPF activity, are generally used, but effective activation methods for reconstructed oocytes vary according to the species. In reconstructed bovine oocytes, electric stimuli, ET, ionomycin, or CaI has been used as the primary activation treatment, and then CHX [4, 5, 20, 21], DMAP [16, 20], or butyrolactone-I [2, 22] are used as the secondary activation treatment. Experiment 1 examined the effects of ET as the primary activation treatment and showed that both the primary and the secondary activation treatments improved the development to blastocysts (11.7%) more than with the primary or the secondary activation treatment alone (1.7% and 0%, respectively). Therefore, both the primary and the secondary activation treatments appear to be beneficial for further development of reconstructed oocytes.

In Experiment 2, we examined the effects of primary activation substances before the secondary activation treatment with CHX. The results showed that ET-CHX (10.5%) would be more beneficial for blastocyst development of reconstructed oocytes than CaI-CHX (4.7%). ET is cheap and can be easily treated with high efficiency instead of electric stimuli, which need special equipment and expensive reagents. ET activates oocytes by promoting the rapid potentiation of inositol 1,4,5-trisphosphate (InsP_3) mediated Ca^{2+} release through stimulation of InsP_3 formation at the plasma membrane [34]. In contrast, CaI stimulates cortical granule release, extrusion of the second polar body, and pronuclei formation and activates oocytes [34], so that the difference between ET and CaI in the proportion of development to the blastocyst stage might be due to differences between the activation processes in reconstructed oocytes.

In Experiment 3, the effects of secondary activation substances after the primary activation treatment with ET were examined. The results showed that ET-CHX (5.9%) and ET-DMAP (2.8%)

did not differ in the proportions of blastocyst development. CHX prevents synthesis of protein on the ribosome, and prevents protein synthesis [20, 21]. As reported that DMAP prevents phosphorylations [20, 21], it is considered that CHX and DMAP maintain the inactivation of MPF. Future studies are needed for the establishment of an appropriate activation method for reconstructed bovine oocytes to improve blastocyst development after ICI.

In conclusion, the present study indicates that the activation treatment with a combination of ET and

CHX could improve blastocyst development of reconstructed bovine oocytes derived by ICI.

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