Bovine Nucleus Transplantation by Intracytoplasmic Injection

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Abstract. This study investigated bovine somatic nucleus transplantation (NT) using an
intracytoplasmic injection system in which cumulus cell nuclei were injected into enucleated oocytes
by a piezo micromanipulator. Activation of NT embryos was carried out by (1) simultaneous injection
of 2–4 pl of inositol 1,4,5 tri-phosphate (IP3) and a donor nucleus into a recipient cytoplasm (IP
method), (2) IP method followed by 1.9 mM 6-dimethylaminopurine (DM) treatment for 3.5 h (IP+DM
method), or (3) activated after NT by an ionomycin treatment (IA) followed by DM treatment (IA+DM
method). We found the frequency of remodeling nuclei and development into blastocyst stage of NT
embryos to be: IP, 44% and 9%; IP+DM, 58% and 29%; and IA+DM, 61% and 27%. These results
indicate that IP3 injection into the intracytoplasm followed by DM treatment produces a similar
bovine oocyte parthenogenetic activation as IA+DM activation treatment, and that intracytoplasmic
nucleus injection is an effective method for bovine somatic NT.

Key words: Nucleus transplantation, Intracytoplasmic injection, Parthenogenetic activation, In vitro-
produced bovine embryo

Following the first report of mammalian somatic nuclear transplantation (NT) by Wilmut et al.
[1], transferring G0/G1 stage nuclei into enucleated oocytes at the second metaphase has successfully
produced clone offspring in cattle [2–4]. In fact, the NT method is considered as a suitable system for
producing (i) super cattle for the dairy [5] and meat [3, 6] industries, and (ii) transgenic cattle derived
from somatic cells after gene transfer [7, 8]. The production efficiency of such somatic cell-cloned
offspring, however, is generally quite low [2–6]; hence, further studies are required to improve the
production efficiency of transferable embryos and offspring using this technique. Although
electrostimulation produces membrane fusion of the transplanted nucleus to the enucleated oocytes
in ordinary bovine NT, it reduces the fusion rate when comparatively small cells are used as the
nucleus donor [9, 10].

Intracytoplasmic injection (ICI) developed by Kimura and Yanagimachi [11] allows the transfer of
nuclei into cytoplasts, and is an alternative method to electrofusion of karyoplasts and cytoplasts [12,
13]. Wakayama [12] used an ICI system to transfer somatic nuclei, ultimately producing viable cloned
mice. Use of this system in bovine somatic cell NT actually makes electrofusion unnecessary; a process
that is known to decrease productivity of NT embryos [9, 10]. Such considerations have led to
applying intracytoplasmic NT in combination with a piezo impact injection system to examine whether
or not the operations/results of bovine NT can be improved.

Activation of oocytes is a major factor responsible for loss in NT efficiency. Standard
electrostimulation is an essential component of
current bovine NT in that it not only induces fusion of the donor nucleus with the host cytoplasm but also induces oocyte activation. As the cell membrane of recipient oocytes undergoes stress due to osmolality shock by ethanol activation treatment and micropores occur due to electrostimulation treatment, another activation method may be suitable for NT embryos whose oolemma are injured by puncturing during ICI [11]. Because Ca-ionophore A23187 and ionomycin treatments have been combined with calcium-elevating agents and a protein synthesis inhibitor for parthenogenetic activation [14], and because inositol 1,4,5 tri-phosphate (IP3) electroporated into matured oocytes [15], or IP3-injected into oocytes [16], produces dynamic changes in intercellular calcium release, we decided to investigate using IP3 as a new activation method for bovine NT embryos. Here, we describe the appropriate conditions for inducing activation and evaluate the effective parthenogenetic activation condition of IP3 on the development of bovine embryos obtained from intracytoplasmic nucleus injection.

Material and Methods

Donor nuclei and recipient cytoplasts
To collect cumulus-oocyte complexes (COCs), follicles from slaughtered Holstein cattle ovaries were transported to our laboratory within 3 h and aspirated through an 18-gauge needle into a disposable 10-ml syringe. Collected COCs were washed three times with 25 mM Hepes buffered TCM-199 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 5% (v:v) FCS (Gibco), 50 µg/ml streptomycin (Meiji Conf. Co. Tokyo Japan), and 100 unit/ml penicillin-G (Meiji). In a single drop of culture medium (0.5 ml) covered with mineral oil (Squib and Sons, Inc., Princeton, NJ, USA), 50–60 COCs were cultured at 38.5 C in humidified air containing 3% CO₂ [17]. After 18 h of maturation culture, COCs were removed by repeated pipetting through a fine-bore pipette in PBS (Gibco) containing 1 mg/ml hyaluronidase (Sigma Co., St. Louis, MO, USA).

Previous studies indicate that NT embryo development is enhanced when donor nuclei are in the G0 or G1 phase [1, 18]. Here, since most cumulus cell nuclei surrounding recently ovulated oocytes were in the G1/G0 phase (more than about 90%), resultant NT embryos had a high development capacity [12, 19, 20] and disaggregated cumulus cells could be used as donor nuclei. These cumulus cells (dia., 7–8 µm) were washed, selected in PBS containing 20% FCS (PB1), and placed in the same medium at room temperature until injected into the recipient cytoplasm.

We selected oocytes with the first polar body as recipient oocytes. Enucleation from recipient oocytes was performed using a previously described procedure [21]. Briefly, matured oocytes were enucleated by aspirating the first polar body and adjacent ooplasm in PB1 containing 5 µg/ml cytochalasin B (Sigma) and 0.1 µg/ml colcemid (Gibco). To confirm removal of the second metaphase chromosomes, fragments of removed ooplasm were stained for 5 min with 5 µg/ml acridine orange (Wako Chemical Co., Tokyo, Japan) and observed using a fluorescence inverted microscope (IMT10: Nikon, Tokyo, Japan). Successfully enucleated oocytes were used as recipient cytoplasts.

Micromanipulator instruments
A micromanipulator (IM-4B: Narishige Co., Ltd., Tokyo, Japan) was attached to an inverted microscope equipped with Hoffman modulation optics (Hoffman Modulation Contrast, Model EP: Nikon). A holding pipette with 130–150 µm outer diameter and 20–30 µm inner diameter was attached to the micromanipulator. Holding pressure was regulated by an air-tight mouth piece connected to the holding pipette by a 40-cm long silicon tube. The tip of the injection needle had an 8-10 µm outer diameter (Sutter Instrument Co., CA, USA). The injection needle was loaded with 4–5 µl of mercury and connected directly to a needle holder that was continuously filled with mineral oil (M-8410, Sigma). An oil-tight syringe (Celltram oil, Eppendorph, AG, Germany) was used to regulate the microinjection. A polyethylene tube connected the syringe to the needle holder, which was also filled with mineral oil. The needle holder was attached to the drive unit of a piezo-micromanipulator (PMM-MB-A: Prime Tech Ltd., Tsuchiura, Japan) that was driven by a controller (PMAS-CT-140). This injection system was attached to another micromanipulator.
Intracytoplasmic donor nuclei injection

Intracytoplasmic nuclear injection was carried out as previously described using the so-called Honolulu method [11, 12, 20]. Briefly, the inner surface of the injection pipette was coated by washing several times with PB1 containing 12% polyvinylpyrrolidone (Sigma). The injection pipette supplying donor cells was inserted deep into the cytoplasm from the slit of the zona pellucida and small mounts of cytoplast were sucked into the pipette. A single piezo pulse was then applied to break the oolemma, after which a single donor cell was expelled into the cytoplasm (Fig. 1).

Activation of NT embryos

After 24–25 h of maturation culture, oocyte activation [10] was performed using one of three methods: (1) 25–400 mM of inositol 1,4,5 tri-phosphate (potassium salt, Sigma) diluted by 0.9% NaCl was microinjected (2–4 pl/oocyte) along with ICI [16] into recipient oocytes (IP); (2) IP3-injected NT embryos were transferred into a microdrop of culture medium containing 1.9 mM 6-dimethylaminopurine (DM, Sigma) at 38.5°C for 4 h. (IP+DM); or (3) NT embryos were exposed for 4 min to ionomycin (5 µM: Cal Biochem, La Jolla, CA) in Ca-free PBS supplemented with 0.1 mg/ml PVA (Sigma), washed 3 times with PB1 and treated with DM [14] (IA+DM). These embryos were used for either nuclei observation or further culture testing.

Karyomorphism examination

After waiting 18 h following activation treatment, NT and parthenogenetic embryos were mounted on slides and fixed in 25% acetic alcohol. Karyomorphism was then determined at least 30 h after fixation by staining with 2% aceto-orcein and examining with a phase contrast microscope at 200 and 400× magnification. Karyomorphism of parthenogenetic oocytes was classified into three groups: (1) second metaphase stage, (2) third...
metaphase stage, or (3) pronucleus stage. The results of karyomorphism of transferred donor nuclei were evaluated and classified into one of three groups [21, 22]: (1) a nucleus with no nuclear membrane, a condensed nucleus, or a spindle formation (condensed chromatin; CC), being the three types of karyomorphology that indicate remodeling by exposing the oocyte cytoplasm to high maturation promotion factor activity [18, 23, 24]; (2) a nucleus swollen and developed into a pronucleus-like stage (nuclear swelling; NS), being a morphology considered as a definite indicator of nuclei reprogramming [21, 23, 24]; and (3) a nucleus showing no change in karyomorphism in comparison with the donor nucleus, or a nucleus showing no evidence of either nuclear swelling or condensed chromatin (no evidence; NE).

Embryo culture

NT embryos were cultured for 5 d in pre-equilibrated drops of culture medium (0.5 ml) under the same culture conditions (Day 0=nucleus transplantation). After 5 d the embryos were transferred into microdrops of culture medium containing 100 mM β-mercaptoethanol (Sigma) [25]. Finally the developmental rate to blastocyst stage was determined at day 8. Development ability of NT embryos was assessed by examining in vitro cultures.

Experiment 1

The adopted activation condition was evaluated by karyomorphism of bovine oocytes 18 h after injection of 25–400 mM IP3.

Experiment 2

Synchronization of the developmental stage between the recipient cytoplasm and transferred nuclei was evaluated by examining karyomorphism of embryos 18 h after donor nucleus injection into oocytes with an original nucleus, i.e., NT embryos ended up with an original and transferred nuclei. The karyomorphism of the donor nucleus transferred into oocytes with original nuclei was also compared to that of nuclei transferred into enucleated oocytes.

Experiment 3

In vitro development of NT embryos using the three activation methods was examined. Resultant blastocysts developed from NT embryos were fixed using an air-dry-method and total cell numbers of these blastocysts were determined.

Data analysis

The experiments were repeated three times (n=3) for each group. Statistical significance was evaluated using the chi-squared test in which P <0.05 was considered statistically significant.

Results

About 50 NT embryos could be manipulated in 1 h using a series NT process. Of note, the piezo pulse punctured the oolemma of 14 (6%) embryos. Karyomorphism examinations showed that ICI NT operations do not produce NT embryos with multiple nuclei or without a nucleus, i.e., all transplanted nuclei were remodeled into either CC or NS karyomorphology. This indicates that the ICI method can suitably transfer nuclei into a recipient cytoplasm.

Figure 2 shows the karyomorphism of oocytes 18 h after parthenogenetic activation by injection of 25–400 mM IP3, where the activation rate of oocytes was affected by increasing IP3 concentration. Note that the activation rate of oocytes injected with 200 or 400 mM IP3 is the same as that for the IA+DM treatment.

Figure 3 shows the karyomorphism of donor nuclei injected into enucleated oocytes, oocytes with an original nucleus, and parthenogenetic nuclei from recipient oocytes 18 h after activation. Note that the high frequency (71–81%) of PN formation occurred for original recipient nuclei using these activation methods, whereas that for transferred nuclei was significantly lower in the IP and IP+DM groups (IP, P<0.01; IP+DM, P<0.05). The frequency of PN formation of nuclei injected into oocytes with original nuclei was the same as that of nuclei injected into enucleated oocytes in all groups. Such poor donor nucleus remodeling indicates that donor nuclei cannot synchronize with the cell cycle of recipient oocytes.

Table 1 summarizes results of in vitro development of NT embryos, where the development rate into the blastocyst stage for DM treatment groups was significantly high (P<0.05). Cleavage and blastocyst rates of NT embryos activated by IP+DM were comparable to those
activated by IA+DM (76% and 29%, 86% and 27%, respectively). The average cell number of 23 day-8 blastocysts showing good morphology was 135 ± 34.

**Discussion**

A bovine karyoplast-cytoplast complex is in general fused within its membrane by applying direct-current pulses using two wire electrodes [2, 4, 19] or a pair of micro-electro needles [3, 5, 26]. Electric stimulation, however, has drawbacks because the fusion rate decreases as the diameter of the cell becomes smaller [9, 10], and the fusion rate is different among donor cell types [3, 6]. Of particular interest, no report exists in which electrofusion was carried out with a recipient ooplast and donor cell smaller than 15 µm, although here 8 µm cells were used as the nucleus donor. Application of the ICI method to inject a single nuclei assuredly led to intracytoplasm, as karyomorphism examinations showed no NT embryos had multiple nuclei or were missing a nucleus; results indicating that ICI can...
comparatively simplify and effectively improve bovine somatic NT operations. In fact, the resultant *in vitro* developmental rate to the blastocyst stage was similar to those obtained using conventional NT methods [2–6], as was the average cell number of resultant blastocysts compared to (i) conventional method NT blastocysts [4, 26] and (ii) *in vitro* matured/fertilized/cultured bovine embryos at the same stage [17]. Accordingly, we have demonstrated that ICI is a feasible method for the production of bovine somatic NT embryos.

Electrostimulation simultaneously serves two functions, i.e., donor-recipient membrane fusion and activation of the recipient cytoplasm. ICI, on the other hand, does not have the capability to activate oocytes [11, 12], so it can be intentionally used for NT to introduce a donor nucleus into a cytoplasm at the second metaphase, or to prolong the time from nuclei injection to oocyte activation [12, 20]. Exposure of somatic cell nuclei into enucleated oocytes at the M2 stage is considered to be the most important factor for developmental potency of NT mouse [12] and pig embryos [13, 20]. ICI may also prove useful for elucidating factors governing developmental potency of NT embryos.

It is known that the injection of IP3 and electroporation in an IP3 medium can induce parthenogenetic activation in *rabbit* [15] and bovine [16] M2 stage oocytes. Here we found the activation rate of oocytes is dependent on IP3 concentration in that a 200–400 mM IP3 injection induced oocyte activation and subsequently PN formation, even with simultaneous electrostimulation. Furthermore, development of NT embryos activated by IP+DM to the blastocyst stage was nearly the same as that activated by IA+DM, which indicates that IP+DM treatment can be effectively used for oocyte activation in bovine NT protocol.

Rabbit oocytes activated with electroporation in an IP3 medium do not exhibit repetitive Ca oscillations over several hours, exhibiting instead a single Ca transient [16]. Incomplete activation with a single Ca transient induces the appearance of M3 stage oocytes [16], and long-term M3 stage oocytes induce chromatin damage leading to embryonic fragmentation [24]. We also observed M3 stage embryos and embryonic fragmentation under all activation conditions. Thus, improvement of bovine ICI NT requires another effective parthenogenetic treatment.

In Experiment 2 in which donor nuclei were inserted into oocytes with original nuclei, 20–30% nuclei remaining in a pronuclear stage cytoplast still stopped at CC karyomorphology 18 h after NT, with the frequency of CC karyomorphology being the same as that of transferred nuclei into enucleated oocytes. The same phenomenon in which transferred donor nuclei were condensed was observed in the pig [20] and rabbit [Ogura personal communication]. These results suggest that it is more difficult for ICI nuclei to form pronuclei than original nuclei. Wakayama [12] and Nagashima [20] reported ICI nuclei exhibited dispersion of chromosomes, disarranged chromosomes, and formed multiple nuclei. On the other hand, these structures have not been observed in parthenogenetic [14] or IVF [17] embryos 18 h after treatment. Such karyomorphism was also not seen in nuclei transferred by electrostimulation [26], indicating these nucleus structures might be specifically recognized using the ICI system. It should be noted Wei and Fukui [27] reported that concentration and volume of injection medium surrounding transferred nuclei appeared to disturb nuclei undergoing PN formation due to (i) injection

### Table 1. *In vitro* development of bovine NT embryos after indicated activation treatment

<table>
<thead>
<tr>
<th>Activation treatment</th>
<th>No. of cultured embryos</th>
<th>Embryo development (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Fragment</td>
</tr>
<tr>
<td>IP</td>
<td>86</td>
<td>20^a (23)</td>
</tr>
<tr>
<td>IP + DM</td>
<td>68</td>
<td>6^c (9)</td>
</tr>
<tr>
<td>IA + DM</td>
<td>51</td>
<td>2^b (4)</td>
</tr>
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IP, inositol 1,4,5-triphosphate; DM, dimethylaminopurine; and IA, ionomycin. a< b (P< 0.01), a< c (P< 0.05)
medium staying in the cytoplasm, and (ii) injected nuclei not being exposed to cytoplasmic factor(s) existing in nuclei during PN formation; hence, minor technical changes in the ICI method should improve nuclear remodeling. In either case, additional basic research will have to be carried out on nuclei remodeling in order to improve the developmental potency of bovine NT embryos in the ICI system.

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References


