

Effect of Repetitive Electrical Stimuli on Development of Nuclear Transferred Bovine Embryos

Fumihito Aono^{1*}, Tomohiro Kono², Nobuhiro Shimozawa^{2#},
Isao Ogiwara¹, Fumio Sekizawa³, Mituo Saitoh³, Tatsuo Nakahara²

¹KYODO SHIRYO CO. LTD., Kanagawa 240-0026, ²NODAI Research Institute,
Tokyo University of Agriculture, Tokyo 156-0054, and ³Tochigi Prefectural
Dairy Experimental Institute, Tochigi 329-2747, Japan

[#]Present address: Central Institute for Experimental Animals, Kanagawa 216-0001, Japan

Abstract: The present study was conducted to examine the effect of repetitive electrical pulses applied to recipient ooplasts and donor blastomeres on the development of nuclear transferred bovine embryos. A series of repetitive electrical pulses at 30 min. intervals improved the rates of activated oocytes and the pronuclear formation in oocytes matured in vitro. After fusion with a donor nucleus, more than 20% of the reconstituted embryos developed to the blastocyst stage in vitro. Additional electrical pulses before fusion to the donor blastomeres and recipient ooplasts significantly enhanced the percentage of reconstituted embryos developing to blastocysts (38%). These results suggest that a series of repetitive electrical pulses can clearly induce suitable oocyte activation and enhance the development of nuclear transferred bovine embryos.

Key words: Nuclear transfer, Repetitive electrical stimuli, Activated ooplast, Bovine

In nuclear transfer of bovine embryos, both unactivated and activated ooplasts (enucleated oocytes) can take as their recipients cytoplasts that received a donor nucleus. We have shown that activation of the ooplast greatly influences the development of nuclear transferred bovine embryos when compared to the control [1]. An effective procedure for the induction of oocyte activation is needed to ensure satisfactory development of the oocyte in nuclear transferred embryos. Parthenogenetic activation of mammalian oocytes can be induced by various chemical and physical stimuli that cause an increase in intracellular Ca^{2+} . Since the sen-

sitivity of oocytes to an artificial activation stimulus increases with the age of the oocytes [2, 3], over-matured bovine oocytes have often been used as recipient oocytes in nuclear transfer [1]. But it has been reported that the developmental ability of reconstituted bovine embryos in which over-matured oocytes are used as recipient cytoplasm is definitely inferior to that of reconstituted embryos from properly matured oocytes. The use of repetitive electrical stimuli has been shown to be effective in inducing activation of newly ovulated mouse and rabbit oocytes, and to allow subsequent development to blastocysts and fetuses [4]. Here we evaluate the effect of a series of repetitive electrical stimuli for the induction of oocyte activation on the ability of nuclear transferred bovine embryos to develop.

Materials and Methods

Oocyte activation

The oocytes were activated parthenogenetically by electrical stimulation with an electrical cell fusion processor (Shimadzu Co., Kyoto) as follows. Oocytes were rinsed in a solution of 0.3 M mannitol containing 0.1 mM MgSO_4 , 0.1 mM CaCl_2 , and 0.01 mg/ml BSA [5], and then placed in the same solution in a cell fusion chamber in which were parallel stainless steel wire electrodes 1 mm apart. Oocyte activation was examined 18 h after the first electrical stimulation; the oocytes were placed on a glass slide, fixed with a 3:1 solution of ethanol/acetic acid for 24 h, and stained with 1% aceto orcein. Oocytes with one or two pronuclei were judged to be activated.

Donor embryos

In vivo donor embryos were obtained by superovula-

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*To whom correspondence should be addressed.

tion and nonsurgical recovery. Donor cows were treated with follicle stimulating hormone (FSH) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the mid estrus cycle and inseminated after estrus. The embryos were recovered 5 days after nonsurgical artificial insemination and transported to the laboratory in TCM-199 (25 mM Hepes buffered TCM-199 with Earle's salts; Gibco, New York) supplemented with 5% (v/v) calf serum (Gibco), penicillin-G (100 U/ml, Meiji Co., Tokyo) and streptomycin (50 μ g/ml, Meiji Co.,) [6].

In vitro donor embryos were obtained by *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culturing (IVC). Bovine ovaries were collected from a slaughterhouse and transported to the laboratory in physical saline solution. Oocytes were aspirated from ovarian follicles. The oocytes with tightly packed cumulus cells were matured *in vitro* by culturing for 20 h in TCM-199 medium supplemented with 10% (v/v) calf serum, penicillin-G (100 U/ml) and streptomycin (50 μ g/ml) in an atmosphere of 5% CO_2 and 95% air at 39°C.

To produce donor embryos by IVF, matured oocytes were transferred to the sperm suspension in BO medium supplemented with 5 mM caffeine (Sigma Chemical Co., St. Louis), 5 IU/ml heparin (Wako chemical Co., Osaka) and 10 mg/ml bovine serum albumin (Gibco). After 6 h of insemination, the oocytes were transferred into TCM-199 medium supplemented with 5% (v/v) calf serum, penicillin-G (100 U/ml) and streptomycin (50 μ g/ml), and cultured for 5 days [7]. Embryos at cell stages 16–32 were used as donors for nuclear transfer.

Enucleation and nuclear transfer

Micromanipulation for the enucleation and the nuclear transfer was carried out as described previously [1]. IVM oocytes were used as a source of recipient ooplasm. After 20–22 h of maturation, the cumulus cells of the oocytes were removed by pipetting in PBS (–) supplemented with 300 unit/ml hyaluronidase (Sigma), and these oocytes with a first polar body were used. The oocytes were placed in PBS containing 5 μ g/ml cytochalasin B (Sigma). The zona pellucida of each matured oocyte was incised along 30% of its circumference, close to the first polar body, with a glass needle. Removal of the Metaphase II (MII) chromosomes was accomplished by aspiration of one-sixth to one-eighth of the cytoplasm near the polar body with an enucleation pipette. To ensure enucleation, the aspirated ooplasm, containing its spindle and the MII chromosomes, was stained with Hoechst 33342 (Sigma) and observed by fluorescent microscopy. The zona pellucida of donor embryos were cut and removed with a glass needle. The zona-free

embryos were exposed to Ca^{2+} free mPBS supplemented with 0.2% EDTA (Sigma) and 0.025% trypsin (Sigma) for a few minutes, and the blastomeres were separated into single cells by gentle pipetting. Donor blastomeres were then inserted into the perivitelline space of the recipient ooplasm.

To induce cell fusion, the aggregation plate of the ooplasm and donor blastomere was orientated in parallel with electrodes by means of an alternating current and a single DC pulse (1.0 kv/cm, 75 μ sec) at 34 hpm. A solution of 0.3 M mannitol containing 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.01 mg/ml BSA [7], and a cell fusion chamber, which were used for oocyte activation, were used for cell fusion.

In vitro culture after nuclear transfer

The fused oocytes were co-cultured with the fibroblast cells derived from mouse fetuses in TCM-199 medium supplemented with 5% (v/v) calf serum, penicillin-G (100 U/ml) and streptomycin (50 μ g/ml) for 7–9 days in an atmosphere of 5% CO_2 and 95% air at 39°C. Mouse fetus fibroblast cells were prepared as described previously [8]. All data were analyzed by Chi-squared analysis.

Results

Experiment 1

To assess the efficiency of a given series of repetitive electrical stimuli, we first examined the activation rates of oocytes with 3 stimulus schedules (Table 1): Group 1 oocytes received five direct current (DC) pulses at intervals of 1 sec (0.75 kv/cm, 30 sec) at 30 hours post maturation (hpm); Group 2 oocytes received the same series of five pulses at 30, 30.5 and 31 hpm; and Group 3 oocytes received the same series at 30, 30.5, 31 and 33 hpm. Of the oocytes receiving five DC pulses at 30 hpm (Group 1), 78% were activated based on the formation of a 2nd polar body and a pronucleus. The activation rates were enhanced in oocytes that received the electrical stimulus repetitively. In Group 3, all examined oocytes were activated and had formed pronuclei. Of the activated oocytes in Group 3, 38% developed to blastocysts *in vitro*.

Experiment 2

In the second experiment, we examined the ability of nuclear transferred embryos to develop to blastocysts *in vitro* (Table 2). The ooplasm received five direct current (DC) pulses at intervals of 1 sec (0.75 kv/cm, 30 sec) at 30, 30.5 and 31 hpm. After injection of a donor

Table 1. Activation of nonaged bovine oocytes by repetitive electrical stimuli

Electrical stimulus at		No. of oocytes examined	No. of oocytes which formed pronuclei (PN) (%)			
			1PN	2PN	3PN	total
Control	non	34	2 (6)	0 (0)	0 (0)	2 (6) a
Group 1	30 hpm*	55	9 (16)	34 (62)	0 (0)	43 (78) b,c
Group 2	30,30.5,31 hpm	60	13 (22)	42 (70)	1 (2)	56 (93) b,d
Group 3	30,30.5,31 33 hpm	50	10 (20)	40 (80)	0 (0)	50 (100) b,d

*Hours post maturation.

The figures with different superscripts in each column are significantly different (a,b: $P<0.001$, c,d: $P<0.05$).**Table 2.** Development of bovine nuclear transplants activated by repetitive electrical stimuli

Origin of donors	Electrical stimulus at	Fused at	No. of oocytes examined	No. of oocytes fused (%)	Development to (%)		
					2-cell	8-cell	Blastosyst
<i>In vitro</i>	30, 30.5, 31 hpm*	33 hpm	143	123 (86)	95 (77)	40 (33)	30 (24) a
	30, 30.5, 31 33 hpm	34 hpm	147	119 (81)	97 (82)	56 (47)	47 (39) b
<i>In vivo</i>	30, 30.5, 31 hpm	33 hpm	31	30 (97)	21 (70)	12 (40)	7 (23)
	30, 30.5, 31 33 hpm	34 hpm	88	82 (93)	70 (85)	35 (43)	31 (38)

*Hours post maturation.

The numbers with different subscripts (a,b) in each column are significantly different ($P<0.05$).**Table 3.** Effect of an additional electrical stimulus applied to donor blastomeres or recipient ooplasts on development of nuclear transplants

Electrical stimuli to	No. of oocytes examined	No. of oocytes fused (%)	Development to (%)		
			2-cell	8-cell	Blastosyst
Donors and recipients	102	83 (81)	68 (82)	39 (47)	31 (37)
Donor nuclei	67	63 (94)	44 (70)	28 (44)	22 (35)
Recipient ooplast	57	53 (93)	37 (70)	23 (43)	20 (38)

blastomere into the perivitelline space of each ooplast, a single DC pulse (1.0 kv/cm, 75 μ sec) was applied to the donor nucleus and the recipient ooplast at 33 hpm. Ooplasts and donor blastomeres were orientated at right angles with electrodes to prevent fusion. The electrical stimulus used for fusion was a single DC pulse at 34 hpm.

A single electrical pulse (1.0 kv/cm, 75 μ sec) given 1h before fusion with a donor nucleus, improved the ability of nuclear transferred embryos to develop. The results showed that a higher proportion of the embryos developed to blastocysts in Group 3 than in Group 2 (39% vs. 24% in *in vitro* donors, 38% vs. 23% in *in vivo* donors, respectively). The numbers of cells at blastocysts were counted to assess their quality, and no significant differences were found between groups.

or the recipient ooplast enhanced the ability of nuclear transferred embryos to develop. To evaluate the effects of electrical stimuli on either the donor cell or recipient ooplast, a single electrical pulse was applied separately to the donor cell or ooplast before reconstitution at 33 hpm, and then the blastomere was fused with the ooplast by means of a single DC pulse (1.0 kv/cm, 75 μ sec) at 34 hpm. The percentage of reconstituted oocytes that had developed to blastocysts was quite similar in the two groups (Table 3), suggesting that applying an electrical stimulus to the donor blastomere enhanced the ability of the nuclear transferred embryos to develop. To reveal the ability of the nuclear transferred embryos, 10 blastocysts were transferred to recipients cows, and two offspring were obtained.

Discussion

Experiment 3

Additional electrical pulses applied to the donor cell

We have shown that the activation of recipient

ooplasts prior to fusion with a donor nucleus improves the potential of bovine nuclear transplants to develop [1]. Over-matured oocytes were used to obtain high activation rates, based on the fact that oocytes matured for a short time are barely responsive to an artificial activation stimulus and only become responsive with time, but it has been reported that over-matured oocytes are inferior to properly matured oocytes with respect to development of the nuclear transferred embryos. To induce activation in younger oocytes, a more consideration of three different stimuli—electrical pulses, Ca^{2+} ionophores and cycloheximide—has been proposed [9]. In the present study we tried to establish a nonchemical oocyte activation procedure for young oocytes by using electrical pulses, and examined the efficiency of this procedure in terms of the ability of bovine nuclear transferred embryos to develop. A DC current pulse creates Ca^{2+} transients that induce Ca^{2+} to enter the oocyte through pores created in the membrane by the stimulus. Ozil [4] has reported that both the activation and the development of parthenogenetic rabbit eggs, including young oocytes, were enhanced by applying a series of repetitive electrical stimuli. We here showed the efficacy of a series of repetitive electrical stimuli for induction of artificial activation in unaged bovine oocytes. When the procedure was used for nuclear transfer, the ability of the nuclear transplants to develop was significantly improved: in repeated trials, we consistently found that 38% of the nuclear transplants developed to the blastocyst stage. Compared with the results obtained so far, the improved development was quite evident, a constant 30–40% of the nuclear transplants developing to the blastocyst stage.

In preliminary experiments, we accidentally discovered that those reconstituted embryos that failed to fuse with a donor nucleus in the first trial, but completed it in the second trial, showed signs of better development to the blastocyst stage. This suggests that applying an electrical stimulus to donor nuclei may improve the ability of nuclear transplants to develop. We therefore tested the effect of electrical pulses applied to the donor nucleus on the development of nuclear transplants. The development of the nuclear transplants, in which the donor nucleus received an electrical stimulus before fusion, was significantly improved. These results, however, do not entirely clarify the effects of electrical pulses on donor nuclei. That is, the reason why electrical pulses applied to donor nuclei improved the ability of nuclear transplants to develop remains unclear. A study on mouse osteoblast-like cells has shown that electrical pulses increase DNA synthesis *via* an increase in the

intracellular Ca^{2+} concentration [10]. The results suggest that more suitable activation procedures with electrical pulses can be developed, and that additional stimulation of reconstituted embryos to increase the intracellular Ca^{2+} concentration might further improve their ability to develop.

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