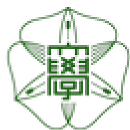

Title	Effect of activation treatments of recipient oocytes on subsequent development of bovine nuclear transfer embryos
Author(s)	Atabay, Edwin C.; Katagiri, Seiji; Nagano, Masashi; Takahashi, Yoshiyuki
Citation	Japanese Journal of Veterinary Research, 50(4), 185-194
Issue Date	2003-02-28
DOI	10.14943/jjvr.50.4.185
Doc URL	http://hdl.handle.net/2115/2966
Type	bulletin (article)
File Information	KJ00000699559.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Jpn. J. Vet. Res. 50(4): 185-194, 2003

FULL PAPER

Effect of activation treatments of recipient oocytes on subsequent development of bovine nuclear transfer embryos

Edwin C. Atabay, Seiji Katagiri, Masashi Nagano and Yoshiyuki Takahashi*

(Accepted for publication : January 9, 2003)

Abstract

Effects of recipient oocyte activation methods on the development of nuclear transfer (NT) embryos were investigated. In Exp. 1, cell-cycle phase of serum-starved bovine cumulus cells was examined by flow cytometry. Majority (95.5%) of medium-sized (16-20 μm) cells that made up 56% of total cells was at the G_0/G_1 phase. NT embryos were constructed by electric fusion with the medium-sized serum-starved cumulus cells and bovine oocytes of 3 different preparations: enucleated oocytes treated with calcium ionophore A 23187 for 5 min and cycloheximide for 5 hr (A 23187/CHX), those treated with ethanol for 7 min and cycloheximide for 2 hr (ethanol/CHX) and those without treatment. In Exp. 2 and 3, developmental competence of NT embryos constructed with A 23187/CHX- and ethanol/CHX-treated oocytes was compared to that of NT embryos constructed with non-treated oocytes, respectively. Further, nuclear behavior in 3 different NT embryos was examined in Exp. 4. Within 1 hr after fusion, majority of the NT embryos constructed with non-treated oocytes showed condensed chromosome. Three hours after fusion, about 50% of NT embryos constructed with non-treated or ethanol / CHX-treated oocytes showed a single pronucleus-like structure. NT embryos constructed with ethanol/CHX-treated oocytes showed similar rates of fusion, cleavage and blastocyst formation to those of the non-treated oocytes. In contrast, NT embryos constructed with A 23187 / CHX-treated oocytes did not show any pronucleus-like structure and showed lower cleavage rate and no development to blastocysts. The results indicate that ethanol/CHX-treated oocytes could support development of somatic cell NT embryos to the blastocyst stage at a

Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
*Corresponding author : Yoshiyuki Takahashi
Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
Tel & Fax : +81-11-706-5231
E-mail address : ytaka@vetmed.hokudai.ac.jp

similar rate to that of non-treated oocytes.

Key words: ethanol; calcium ionophore; cycloheximide; cumulus cells

Introduction

Bovine somatic cell-derived NT embryos have been produced using enucleated mature oocytes at metaphase II (cytoplasts) and somatic cells (nuclear donor cells) arrested at the G_0/G_1 phase[8,9,14,23,25]. Recent studies demonstrated that somatic cell NT embryos can also be produced by using activated oocytes[5,10,11]. In these studies, the metaphase II oocytes were activated with chemicals to release the oocytes from meiotic arrest and then enucleated at telophase II (2 - 5 hr after activation)[5,11]. Enucleation at telophase II removes chromatin materials by aspirating the second polar body and surrounding cytoplasm; thus, enables more efficient enucleation (92-98%) than the enucleation at metaphase II (59-62%)[4,11,15]. This method has further advantages since enucleation can be done with minimal removal of cytoplasm and without exposure to DNA stain or UV irradiation for chromatin localization. Moreover, enucleation at the telophase II stage enables selection of a highly homogeneous group of synchronously activated host oocytes[4].

The NT embryos constructed with activated oocytes and G_1/S phase[10], G_1 phase[11] and cyclic cells[5] possess developmental competence similar to or better than those constructed with non-activated metaphase II oocytes and G_0/G_1 phase cells[5,10,11]. Furthermore, the pregnancy in cattle[5] and birth of live offspring in goats[2] have been reported using activated and telophase-enucleated oocytes. However, developmental competence of NT embryos with activated oocytes seems controversial. Some studies showed no development of NT embryos with activated oocytes to the blastocyst stage[20]. Thus, developmental competence of NT embryos constructed with activated oocytes remains to be confirmed.

It has been accepted that efficiency of oocyte activation and developmental competence of NT embryos constructed with activated oocytes are affected by various factors, such as activating agents, the duration between initial activation stimulus and fusion to the nuclear donors, the age (*i.e.*, time after maturation culture) of the oocytes at activation, cell-cycle coordination between nuclear donors and recipient oocytes[6]. Although every protocol used for oocyte activation in recent NT embryo production differs in details, there are two typical protocols used for oocyte activation. One protocol treats oocytes with 5-20 μM ionophores for 5 min followed by 10 μg/ml cycloheximide for 5 - 6 hr till fusion to nuclear donors[15]. The other treats oocytes with 7 % ethanol for 5 min followed by incubation for 2 - 3 hr with or without cycloheximide or 6-dimethylaminopurine[10].

Therefore, we compared the developmental competence of NT embryos constructed with metaphase II oocytes activated by the two typical protocols to that of the NT embryos constructed with non-activated metaphase II oocytes fused and activated simultaneously in this study. To exclude the effect of the enucleation procedure and cell-cycle phase of the nuclear donors, oocytes were first enucleated, then activated with different chemicals and fused with nuclear donors at the same cell-cycle phase.

Morphological changes of transplanted nuclei in the cytoplasts give an insight of the activities of maturation promoting factor (MPF) and cytostatic factor (CSF)[6], key com-

ponents of cell cycle-phase regulation. Thus, we also investigated the morphological changes of transplanted nuclei in the recipient cytoplasts to understand the difference in the developmental competence of NT embryos reconstructed with oocytes prepared with different activation procedures.

Materials and Methods

Preparation of recipient oocytes

In vitro matured bovine oocytes were used as recipient cytoplasts for NT. They were prepared as described previously[18]. Briefly, cumulus-oocyte complexes (COCs) aspirated from small antral follicles of slaughterhouse-derived ovaries were cultured for 18 to 20 hr at 39℃ under a humidified air with 5 % CO_2 in a maturation medium: 25 mM HEPES-buffered TCM 199 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco), 0.02 units / ml follicle stimulating hormone (Sigma Chemical Company, St. Louis, MO, USA), 1 μg/ml estradiol-17β (Sigma), 0.2 mM sodium pyruvate (Sigma) and 50 μg/ml gentamicin sulfate (Sigma).

Enucleation of oocytes was done as described previously[1]. Briefly, after the removal of cumulus cells by repeated pipetting and short time of vortexing, cumulus-free oocytes were mechanically enucleated by removing the first polar body and approximately 20% of the adjacent cytoplasm in HEPES-buffered modified Tyrode's medium (TALP-HEPES)[3] supplemented with 10% FCS, 0.2 mM sodium pyruvate and 5 μg/ml cytochalasin B (Sigma).

Some of the enucleated oocytes were exposed to 20 μM calcium ionophore A 23187 (Sigma) for 5 min in an embryo culture medium : a modified synthetic oviductal fluid containing 20 amino acids and 10 μg/ml insulin[18], and further supplemented with 5 mM glycine, 5 mM taurine, 1 mM glucose and 1 mg/ml polyvinyl alcohol[19]. They were then incubated in the same embryo culture media containing 3 mg/ml fatty acid-free BSA instead of 1 mg/ml polyvinyl alcohol and 10 μg/ml cycloheximide (Sigma) for 5 hr (A23187/CHX)[15] or to 7% (v/v) ethanol for 7 min and cycloheximide for 2 hr (ethanol/CHX)[10] before fusion in an embryo culture medium with 3 mg/ml fatty acid-free BSA at 39℃ in 5 % CO_2, 5% O_2 and 90% N_2.

Preparation of nuclear donor cells

Primary cultured cumulus cells were prepared as described previously[14] with some modifications. Briefly, cumulus cells collected from *in vitro* matured oocytes were cultured in Dulbecco's modified Eagle medium : nutrient mixture F-12 (DMEM/F12, Gibco) supplemented with 10% FCS and 50 μg/ml gentamicin sulfate for 3 or 4 days at 37℃ under a humidified air with 5% CO_2. To obtain the cells at G_0/G_1 phase, the medium was replaced with DMEM/F12 supplemented with 0.5% FCS (serum-starvation) and further cultured for 3 days. Serum-starved cells were disaggregated with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 0.1% (w/v) trypsin (Sigma) and0.1% (w/v) EDTA (Kanto Kagaku, Tokyo, Japan) and were used as nuclear donor cells. The serum-starved cells were characterized in viability, cell size and cell-cycle phase as previously described[14]. Briefly, the cells stained with 0.3% (w/v) trypan blue were counted to examine the viability using a haemocytometer under a light microscope. To determine the cell-size and cell-cycle phase distribution, the diameter of cells were measured using an ocular scale under an inverted microscope. The cells were fixed in cold 70% (v/v) ethanol and analyzed DNA content to examine the cell-cycle phase distribution by flow cytometry.

Fixed cells were washed with DPBS and incubated with 200 μg/ml RNase A (Boehringer Mannheim, Mannheim, Germany) for 30 min at 37℃, stained with 50 μg/ml propidium iodide (Sigma) and filtered through a nylon mesh with 50 μm pores (Kyoshin Riko, Tokyo, Japan). Fluorescence was measured with flow cytometer (EPICS XL-MCL, Beckman Coulter Inc., Fullerton, CA, USA) using a 620 nm filter.

Nuclear transfer and culture of embryos

Nuclear donor cells were inserted individually into the perivitelline space of enucleated oocytes. Serum-starved cells with 16-20 μm in diameter (presumptive G_0/G_1 phase) were fused with non-treated, A 23187/CHX- or ethanol/CHX-treated oocytes. The couplets were placed and manually aligned between two wire electrodes (1.0 mm apart) overlaid with 0.3M mannitol solution containing 0.1 mM $CaCl_2$ and 0.1mM $MgCl_2$. Fusion of couplets was induced by two direct current pulses of 100 V/mm for 40 μsec, 1 sec apart using an Electro Cell Fusion (LF-100, Life Tec Co., Tokyo, Japan). Successfully fused couplets with A23187/CHX-treated oocytes were cultured immediately in the embryo culture medium; whereas, fused couplets with ethanol/CHX-treated and non-treated oocytes were cultured in the embryo culture medium for 1 hr, and thereafter with 10 μg/ml cycloheximide for 5 hr at 39℃ in 5% CO_2, 5% O_2 and 90% N_2. To determine the development to the blastocyst stage, fused couplets were incubated in the embryo culture medium for 7 days at 39℃ in 5% CO_2, 5% O_2 and 90% N_2. The cleavage and development to the 16-32-cell and blastocyst stages were examined 33, 72 and 174 hr after fusion, respectively. The cell numbers of blastocysts were counted at 174 hr after fusion as described previously[17].

Evaluation of morphological status of transplanted nuclei

After fusion of nuclear donor cells and recipient oocytes, NT embryos were fixed with ethanol : acetic acid (3 : 1, v/v) for 24 hr and stained with 1% (w/v) aceto-orcein to determine the transplanted nuclear status under a phase-contrast microscope.

Experimental studies

In experiment 1, the cell size and the cell-cycle phase distribution of primary cultured cumulus cells after serum-starved culture were investigated.

In experiment 2, the *in vitro* development of NT embryos constructed with medium-sized nuclear donor cells (G_0/G_1 phase) and recipient oocytes with or without calcium ionophore A23187 treatment was determined.

In experiment 3, the developmental competence of NT embryos constructed with medium-sized nuclear donor cells and recipient oocytes treated with ethanol / CHX or non-treated oocytes was compared.

In experiment 4, NT embryos derived from medium-sized nuclear donor and nuclear recipients used in experiments 2 and 3 were cultured for 0.5 (except for ethanol / CHX NT embryo), 1 or 3 hr after fusion and evaluated for the nuclear status.

Statistical analysis

Differences between 2 and 3 means were compared using Student's t-test and analysis of variance, respectively. Data analyses were carried out using StatView (Abacus Concepts Inc., Berkeley, CA, USA).

Results

In experiment 1, majority of the serum-starved cells was within the medium size. DNA analysis revealed that 95.5% of the

Table 1. Cell size and cell-cycle phase distribution of bovine primary cumulus cells after serum-starvation culture[a]

Cell size (μm)	%[b] of cells in each size	% of cells in each cell-cycle phase		
		G_0/G_1	S	G_2/M
Small (≤15)	24.0±7.9[c]	99.0±0.2[c]	0.5±0.1[c]	0.5±0.1[c]
Medium (16-20)	55.7±3.2[d]	95.5±1.0[c]	2.0±0.6[d]	2.6±1.1[c]
Large (≥21)	20.3±10.0[c]	80.8±3.5[d]	4.2±0.8[e]	15.5±3.1[d]

[a]Cells were cultured for 3 days with 0.5% fetal calf serum.

[b]One hundred cells were measured per replicate.

[c-e]Values (mean±SD of 3 replicates) with different superscript in a column within a treatment differ significantly ($P<0.05$).

Table 2. *In vitro* development of NT embryos constructed with donor cells at G_0/G_1 phase and A23187/CHX-treated or non-treated oocytes

Recipient[a] oocyte treatment	No. of couplets	% of fused couplets	%[b] of NT embryos develop to		
			≥ 2-cell	≥16-cell	blastocyst
Non-treated	45	63.7±5.1	66.5±5.0[c]	48.2±6.6[c]	33.5±5.0
A23187/CHX	45	55.7±7.6	52.4±6.9[d]	8.5±7.5[d]	0

[a]Non-treated : enucleated oocytes were fused with nuclear donor cells and incubated with cycloheximide for 5 hr ; A23187/CHX : enucleated oocytes were exposed to calcium ionophore A23187 for 5 min then incubated with cycloheximide for 5 hr before fusion.

[b]% based on the number of fused couplets. Development of nuclear transfer (NT) embryos to ≥2-cell, ≥16-cell and blastocysts was determined at 33, 72 and 174 hr after fusion, respectively.

[c,d]Values (mean±SD) of 3 replicates with different superscripts within a column differ significantly ($P<0.05$).

medium-sized cells were at the G_0/G_1 phase after serum starvation (Table 1). Percentages of live cells of the serum-starved cells were 94.7±2.0 (mean±SD of 3 replicates).

In experiment 2, as shown in Table 2, there was no difference in fusion rate, regardless of the recipient oocyte treatment. However, the NT embryos constructed with A23187/CHX-treated oocytes showed lower cleavage and development beyond the 16-cell stage than those constructed with non-treated oocytes ($P<0.05$). No blastocyst was observed in the NT embryos derived from A23187/CHX-treated oocytes.

In experiment 3, as shown in Table 3, there were no significant differences in the rates of fusion, cleavage and development to the blastocyst stage between the NT embryos derived from the oocytes treated with ethanol/CHX and those from non-treated oocytes. However, the mean cell number of blastocysts derived from non-treated oocytes was larger than that from ethanol/CHX-treated oocytes ($P<0.05$).

In experiment 4, at 0.5 hr after fusion, some of the NT embryos (3/9) constructed with non-treated oocytes had condensed chromosome masses; while, all of the NT embryos

Table 3. *In vitro* development of NT embryos constructed with donor cells at G_0/G_1 phase and ethanol/CHX-treated or non-treated oocytes

Recipient[a] oocyte treatment	No. of couplets	% of fused couplets	%[b] of NT embryos develop to			Blastocyst cell no. (n)
			≥ 2-cell	≥16-cell	blastocyst	
Non-treated	58	67.2±0.9	79.5±0.8	38.6±2.5	33.0±2.9	175.3±7.0[c] (13)
Ethanol/CHX	76	69.7±2.7	67.5±10.8	38.6±4.2	30.3±3.6	165.8±12.6[d] (16)

[a]Non-treated: enucleated oocytes were fused with nuclear donor cells and incubated with cycloheximide for 5 hr; Ethanol / CHX: oocytes were exposed to ethanol for 7 min then incubated with cycloheximide for 2 hr before fusion.

[b]% data were mean ± SD of 3 replicates based on the number of fused couplets. Development of nuclear transfer (NT) embryos to ≥2-cell, ≥16-cell and blastocysts was determined at 33, 72 and 174 hr after fusion, respectively.

[c,d]Values (mean ± SD) with different superscripts within a column differ significantly ($P < 0.05$).

Table 4. Changes in the nuclear status of the nuclear transfer embryos constructed with donor cells at G_0/G_1 phase and recipient oocytes with or without treatment

Recipient oocyte treatment[a]	Hours after fusion	No. of couplets examined	No. of couplets with		
			Intact	CCM (≥2CCM)	PN (2 PN)
Non-treated	1	11	2	7 (6)	2 (0)
	3	9	0	1 (0)	8 (4)
A23187/CHX	1	15	13	2 (1)	0
	3	8	6	2 (2)	0
Ethanol/CHX	1	9	7	2 (0)	0
	3	10	2	3 (1)	5 (0)

[a]For explanation of non-treated, A23187/CHX and Ethanol/CHX, see footnotes in Tables 2 and 3.

Intact: presence of nuclear envelope; CCM: condensed chromosome mass; PN: pronucleus-like structure

(10/10) constructed with A23187/CHX-treated oocytes possessed nuclei with intact envelop. As shown in Table 4, more than half of the NT embryos constructed with non-treated oocytes showed one or more condensed chromosome masses, and some embryos showed a single pronucleus-like structure at 1 hr after fusion. A few NT embryos constructed with A 23187/CHX-treated and ethanol/CHX-treated oocytes showed condensed chromosomes but none of both NT embryos showed pronucleus-like structure at 1 hr. At 3 hr after fusion, the NT embryos constructed with non-treated or ethanol/CHX-treated oocytes exhibited pronucleus-like structure, whereas the NT embryos constructed with A23187/CHX-treated oocytes did not show the pronucleus-like structure.

Discussion

NT embryos constructed with non-treated and ethanol/CHX-treated oocytes developed to the blastocyst stage at similar rates while none of NT embryos with A 23187/CHX-treated oocytes developed to blastocysts. The present results confirmed the findings of previous works[10,11,20] and suggest that the activated oocytes with an ethanol/CHX protocol supports development of NT embryos constructed with somatic cells. Thus, the protocol can be used to activate oocytes for telophase enucleation that has greater potential in recipient oocyte preparation than oocyte enucleation at metaphase II[4].

Treatment for oocyte activation aims to inactivate both MPF and CSF. In an intact oocyte, inactivation of MPF is parallel to meiotic release, and inactivation of mitogen-activated protein kinase, a key component of CSF activity, is associated with pronuclear development[6,12]. The transplanted nuclei in the NT embryos constructed with non-treated oocytes may be exposed to high MPF activity since a high proportion of couplets showed condensed chromosomes. These nuclei may form a single pronucleus-like structure as MPF and CSF activities decreased, then enter mitosis. NT embryos which exhibited such nuclear changes may have reprogrammed and acquired developmental competence to the blastocyst stage.

About 50% of NT embryos with non-treated oocytes formed 2 pronucleus-like structures. It has been suggested that the levels of MPF and the duration during which introduced nuclei are exposed to MPF activity determine the number of pronucleus-like structure[20]. In mice, metaphase II oocytes with low CSF and MPF levels form one pronucleus and enter the first mitotic cleavage while those with high MPF levels, due to insufficient inactivation of CSF, form 2 pronuclei and enter metaphase III stage[21,22]. Accordingly, formation of 2 pronucleus-like structures occurred only in NT embryos constructed with non-treated oocytes. While NT embryos with activated oocytes, in which CSF and MPF activity can be assumed at low levels, formed only a single pronucleus-like structure.

The present results highlighted an advantage of the ethanol/CHX protocol over the A23187/CHX protocol in oocyte activation. The difference in the developmental competence of the NT embryos constructed with oocytes activated by the 2 protocols may be explained by the different morphological changes of the transplanted nuclei. When nuclei (at G_0/G_1 phase) are placed in enucleated oocytes that have been fully activated (low MPF and CSF activities), the nuclei should form pronucleus-like structures without nuclear envelop breakdown and chromosome condensation and will proceed to the S-phase[6]. NT embryos with such nuclei seem to continue normal kinetics of mitosis and may develop to the blastocyst stage. This may occur in NT embryos constructed with ethanol/CHX-treated oocytes that showed a single pronucleus-like structure (50%) at 3 hr after fusion but not in those with A23187/CHX-treated oocytes.

It is generally accepted that a series of multiple transient increases in intracellular calcium ion concentrations induced by spermatozoa trigger the activation process of the oocytes[24]. Thus, the difference in the efficiency of the 2 treatments for oocyte activation can partially be attributed to the difference in intracellular calcium responses in the oocytes treated with ethanol and calcium ionophore A23187. Both ethanol (7% for 7 min) and calcium ionophore A23187 (10 μM for 5 min) induce a single rise of intracellular cal-

cium ion concentrations in bovine oocytes and show similar peak levels to that of the first calcium rise induced by fertilization[16]. The rise induced by the ethanol treatment, however, is characterized by a longer duration than the rise induced by calcium ionophore A 23187[16]. The longer duration of the calcium rise in ethanol treated oocytes may better mimic a series of multiple calcium transient increases at fertilization, thus, ethanol treatment makes a better activating stimulus for oocytes than calcium ionophore A23187.

Further, the source of calcium ions may affect the activation efficiency. Calcium ionophore A23187 initiates calcium influx from the extracellular environment and intracellular stores such as smooth endoplasmic reticulum[7]. While ethanol treatment is thought to trigger inositol 1, 4, 5-trisphosphate (IP_3)-mediated calcium rise in Xenopus oocytes, which would be mainly from the intracellular store[7]. The transient rises in intracellular calcium ion concentrations following sperm-egg binding are caused by the breakdown of membrane phosphatidylinositol diphosphate with the resultant production of IP_3 [13]. The similarity between ethanol treatment and fertilization in the source of calcium ions for transient rises may be a part of reasons for higher oocyte activating efficiency of ethanol treatment than calcium ionophore A23187.

The difference in the activation efficiency of the 2 treatments can also be attributed to the different use of cycloheximide in each treatment. Ethanol/CHX protocol used cycloheximide for 2 hr after ethanol treatment and for 5 hr after fusion of the enucleated oocytes and nuclear donors. A23187/CHX protocol, however, used cycloheximide for 5 hr after A 23187 treatment but not after fusion. As described previously, the fate of nucleus differs depending on the levels of MPF and CSF activities. Both protocols are designed to lower the MPF activity by either ethanol or A23187 and to maintain the low MPF level with cycloheximide by preventing production of cyclin B, a key component of MPF[6]. The A23187/CHX protocol used in the present study has been used in the previous works with successful activation of bovine oocytes[12,15]. However, the observation of nuclear morphology suggests that A23187/CHX protocol activated oocyte only partially and that MPF activity resumed to high levels after fusion when the fused couplets were cultured without cycloheximide. This hypothesis should be examined in further study with a modification of A23187/CHX protocol by adding cycloheximide after fusion.

The present study demonstrated a clear advantage of ethanol/CHX protocol over A 23187/CHX protocol to prepare activated recipient oocytes for somatic cell NT embryo production. The results suggest that oocyte activation protocol with ethanol and cycloheximide can be used for telophase enucleation. However, the normality of bovine NT embryos constructed with oocytes that have been activated by ethanol/CHX treatment and enucleated at telophase II remains to be determined. To date, no work has been available for full term development of NT embryos with oocytes activated by ethanol/CHX protocol.

Acknowledgements

This study was supported by grant-in-aid for Scientific Research (No. 10556058) from the Japan Society for the Promotion of Science. The authors gratefully acknowledge the staff members of the Ebetsu Meat Inspection Office for their aid of ovary collection.

References

1) Atabay, E. C., Martinez Diaz, M. A., Dochi, O. and Takahashi, Y. 2001. Factors affecting enucleation rates of bovine and porcine oocytes after removal of cumulus

cells by vortexing. *J. Reprod. Dev.*, 47 : 365-371.

2) Baguisi, A., Behboodi, E., Melican, D. T., Pollock, J. S., Destrempes, M. M., Cammuso, C., Williams, J. L., Nims, S. D., Porter, C. A., Midura, P., Palacios, M. J., Ayres, S. L., Denniston, R. S., Hayes, M. L., Ziomek, C. A., Meade, H. M., Godke, R. A., Gavin, W. G., Overstrom, E. W. and Echelard, Y. 1999. Production of goats by somatic nuclear transfer. *Nat. Biotechnol.*, 17 : 456-461.

3) Bavister, B. D., Leibfried, M. L. and Lieberman, G. 1983. Development of preimplantation embryos of the golden hamster in a defined culture medium. *Biol. Reprod.*, 28 : 235-247.

4) Bordignon, V. and Smith, L. C. 1998. Telophase enucleation: an improved method to prepare recipient cytoplasts for use in bovine nuclear transfer. *Mol. Reprod. Dev.*, 49 : 29-36.

5) Bordignon, V. and Smith, L. C. 2002. Bovine embryos reconstructed with somatic cells arrested at G_0/G_1 with metaphase oocytes or cycling with telophase host oocytes show similar developmental potential. *Theriogenology*, 57 : 200 (abstr).

6) Campbell, K. H. S. 1999. Nuclear equivalence, nuclear transfer, and the cell cycle. *Cloning*, 1 : 3-15.

7) Ilyin, V. and Parker, I. 1992. Effects of alcohols on responses evoked by inositol trisphosphate in Xenopus oocytes. *J. Physiol.*, 448 : 339-354.

8) Kato, Y., Tani, T., Sotomaru, Y., Kurokawa K., Kato, J., Doguchi, H., Yasue, H. and Tsunoda, Y. 1998. Eight calves cloned from somatic cells of a single adult. *Science*, 282 : 2095-2098.

9) Kubota, C., Yamakuchi, H., Todoroki, J., Mizoshita, K., Barber, M., Tabara, N. and Yang, X. 2000. Six cloned calves produced from adult fibroblast cells after long-term culture. *Proc. Natl. Acad. Sci., USA.*, 97: 990-995.

10) Kurosaka, S., Nagao, Y., Minami, N., Yamada, M. and Imai, H. 2002. Dependence of DNA synthesis and in vitro development of bovine nuclear transfer embryos on the stage of the cell cycle of donor cells and recipient cytoplasts. *Biol. Reprod.*, 67 : 643-647.

11) Liu, J. L., Wang, M. K., Sun, Q. Y., Xu, Z. and Chen, D. Y. 2000. Effect of telophase enucleation on bovine somatic nuclear transfer. *Theriogenology*, 54 : 989-998.

12) Liu, L. and Yang, X. 1999. Interplay of maturation-promoting factor and mitogen-activated protein kinase inactivation during metaphase to interphase transition of activated bovine oocytes. *Biol. Reprod.*, 61 : 1-7.

13) Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S. and Mikoshiba, K. 1992. Block of Ca^{2+} wave and Ca^{2+} oscillation by antibody to the inositol 1, 4, 5-trisphosphate receptor in fertilized hamster eggs. *Science*, 257 : 251-255.

14) Mohamed Nour, M. S., Ikeda, K. and Takahashi, Y. 2000. Bovine nuclear transfer using cumulus cells derived from serum-starved and confluent cultures. *J. Reprod. Dev.*, 46 : 85-92.

15) Mohamed Nour, M. S. and Takahashi, Y. 1999. Preparation of young preactivated oocytes with high enucleation efficiency for bovine nuclear transfer. *Theriogenology*, 51 : 661-666.

16) Nakada, K. and Mizuno, J. 1998. Intracellular calcium responses in bovine oocytes induced by spermatozoa and by reagents. *Theriogenology*, 50 : 269-282.

17) Takahashi, Y. and First, N. L. 1992. *In vitro* development of bovine one-cell em-

bryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology*, 37 : 963-978.

18) Takahashi, Y., Hishinuma, M., Matsui, M., Tanaka, H. and Kanagawa, H. 1996. Development of *in vitro* matured/fertilized bovine embryos in a chemically medium : influence of oxygen concentration in the gas atmosphere. *J. Vet. Med. Sci.*, 58 : 897-902.

19) Takahashi, Y. and Kanagawa, H. 1998. Effect of oxygen concentration in the gas atmosphere during *in vitro* insemination of bovine oocytes on the subsequent embryonic development *in vitro*. *J. Vet. Med. Sci.*, 60 : 365-367.

20) Tani, T., Kato, Y. and Tsunoda, Y. 2001. Direct exposure of chromosomes to nonactivated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming. *Biol. Reprod.*, 64 : 324-330.

21) Wakayama, T., Tateno, H., Mombaerts, P. and Yanagimachi, R. 2000. Nuclear transfer into mouse zygotes. *Nature Genetics*, 24 : 108-109.

22) Wakayama, T. and Yanagimachi, R. 2001. Effect of cytokinesis inhibitors, DMSO and the timing of oocyte activation on mouse cloning using cumulus cell nuclei. *Reproduction*, 122 : 49-60.

23) Wells, D. N., Misica, P. M. and Tervit, H. R. 1999. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod.*, 60 : 996-1005.

24) Yanagimachi, R. 1994. Mammalian fertilization. In : *The Physiology of Reproduction*, pp. 189-317, Knobil, E. and Neill, J. D. eds., Raven Press, New York.

25) Zakhartchenko, V., Durcova-Hills, G., Stojkovic, M., Schernthaner, W., Prelle, K., Steinborn, R., Muller, M., Brem, G. and Wolf, E. 1999. Effects of serum starvation and re-cloning on the efficiency of nuclear transfer using bovine fetal fibroblasts. *J. Reprod. Fertil.*, 115 : 325-331.