The Effects of Donor Cell Type and Culture Medium on *in vitro* Development of Domestic Cat Embryos Reconstructed by Nuclear Transplantation

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ABSTRACT : In this study we explored the possibility of performing nuclear transfer in the domestic cat and assessed the ability of different culture media to support *in vitro* development of reconstructed cat embryos. Donor somatic cells were derived from cultured cumulus cells or explants of oviduct tissue, and recipient cytoplasts from *in vitro* matured oocytes. A higher percentage of cleavage (84.6% and 86.5%) and development to the morula stage (35.9% and 44.2%) was found when reconstructed embryos receiving cumulus or oviduct cells were cultured in MK1 medium, compared with those cultured in CR1aa (58.7% and 72.5%, 13.8% and 13. 6%, respectively). There was no significant difference between MK1 and CR1aa media with respect to the proportion developing to the blastocyst stage (15.4% and 17.3% vs 6.8% and 8.6%, respectively, $p>0.05$). There was no significant effect ($p>0.05$) of donor cell type (cumulus and oviduct cells) on the rates of fusion (65.0% and 52.5%), cleavage (84.6% and 86.5%), development to the morula (35.9% and 44.2%), and blastocyst (15.4% and 17.3%) stages when reconstructed embryos were cultured in MK1 medium. Similar results were found for the reconstructed embryos cultured in CR1aa medium. These results show that culture medium has a significant impact on the early development of reconstructed cat embryos, whereas donor cell type does not have a significant effect. *(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 8 : 1057-1061)*

Key Words : Cat, Nuclear Transfer, Somatic Cells

INTRODUCTION

Techniques involving fusion of differentiated somatic cells with enucleated oocytes have been used successfully to generate cloned young in several types of domestic animal (Schnieke et al., 1997; Wilmut et al., 1997; Kato et al., 1998; Wakayama et al., 1998, Ogura et al., 2000). Further study in nuclear transfer techniques demonstrated that this technology provides the potential for safeguarding endangered breeds against extinction (Wells et al., 1999).

The application of *in vitro* fertilization techniques in non-domestic cat species has demonstrated the potential role of assisted reproduction techniques (ART) in conservation of endangered cat species (Pope, 2000). Since the successful birth of kittens after transfer of *in vitro*-produced domestic cat embryos (Goodrowe et al., 1988) considerable progress in wild animal conservation has been achieved using similar techniques to produce offspring in wild cat species (Pope, 2000). Encouraging progress in nuclear transfer techniques in farm animals has created the opportunity for producing embryos by transferring somatic cells into enucleated matured oocytes. However, similar work has not been conducted in the domestic cat. If nuclear transfer techniques could be adopted in the domestic cat, they might prove to be valuable model for conservation of wild cat or other endangered animal species.

The present study used two culture media, CR1aa and MK1. CR1aa medium is designed for bovine embryo culture (Rosenkrans et al., 1993), and has been widely used in cultivation of bovine reconstructed embryos (Dominko et al., 1999). MK1 medium is modified Earle's balanced salt solution, designed for cat embryo culture (Kanda et al., 1988). We chose to compare these media in culturing cat reconstructed embryos because of the similarities of their components.

The present study was thus conducted to investigate the possibility of performing nuclear transfer in domestic cat using somatic cells as donor cells, and to examine the effects of donor cell type and different culture media on the subsequent *in vitro* development of reconstructed cat embryos.

MATERIALS AND METHODS

Derivation of somatic cells

Feline somatic cells for nuclear transfer were obtained by culture of explants from cat oviduct following ovariohysterectomy at local veterinary clinics. Oviduct samples were manually cut into small pieces, then were washed two times in culture medium (Dulbecco's Modified Eagle medium, Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS) and 50 $\mu g \cdot mL^{-1}$ gentamicin (Sigma, St. Louis, MO, USA)] by centrifuging at 500 xg for min, and cultured at 38.5°C in 5% CO_2 in an atmosphere of air. After 10 to 14 days of culture, confluent cells were obtained. The confluent cells were routinely passaged or either frozen in culture medium containing 5% (v/v) dimethylsulfoxide (DMSO, Wako Pure

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Chemical, Osaka, Japan) for further use. In this study, all donor cells for nuclear transfer were prepared from somatic cells cultured from one to three passages. After 72 h of routine culture, confluent cells were transferred to a culture medium containing 0.5% FBS for three to five days before use in nuclear transfer. Cultured cumulus cells were collected from cumulus-oocytes complexes (COCs) and cultured as oviduct cells.

Preparation of recipient oocytes

In vitro maturation of oocytes : Ovaries were collected from domestic cats at various stages of the estrous cycle by ovariohysterectomy at local veterinary clinics. Ovaries were brought to the laboratory in physiological saline (0.85% [w/v] NaCl) at 37 to 39°C within 2 h of removal. The ovaries were washed in modified phosphate-buffered saline (PBS) (Embryotech; Nihonzenyaku, Fukushima, Japan) supplemented with 50 μg mL^{-1} gentamicin at room temperature, and then sliced repeatedly to release oocytes and surrounding cumulus cells. Liberated oocytes exhibiting uniform, homogeneously pigmented ooplasm and an intact cumulus cell investment were chosen and subjected to further treatments. The COCs were washed three times in a maturation medium (TCM199 medium [Earle's salts]) buffered with 25 mM HEPES buffer (Gibco, Grand Island, NY, USA) supplemented with 0.4% bovine serum albumin (BSA, Sigma), 0.1 IU mL^{-1} human menopausal gonadotropin (Teikokuzoki, Tokyo, Japan), 10 IU mL^{-1} human chorionic gonadotropin (Teikokuzoki, Tokyo, Japan), 1 μg mL^{-1} β-estradiol (Sigma) and 50 μg mL^{-1} gentamicin (Sigma). Five oocytes were transferred into a 100 μL drop of maturation medium in a culture dish (35×10 mm; Falcon, Lincoln Park, NJ, USA) overlaid with mineral oil, and incubated for 24 h at 38.0°C in 5% CO_2 in an atmosphere of air. After culture for 24 h, the cumulus-corona was removed by manual pipetting, and the oocytes with extruded first polar body (metaphase II, MII) were selected as recipient oocytes.

To investigate the effects of culture medium on the *in vitro* development of reconstructed embryos, oocytes with an extruded first polar body were divided into two groups before enucleation. From each group, cytoplasts and embryos reconstructed with either cumulus or oviduct cells were incubated in either CR1aa or MK-1 medium during manipulation, activation and culture.

Enucleation : Oocytes with an extruded first polar body were placed into 1 20-μl drop of manipulation medium (PBS, Gibco) supplemented with 5 μg mL^{-1} cytochalasin B and 0.3% BSA. All micromanipulations were performed at room temperature on the stage of an inverted microscope (Nikon Co., Ltd, Japan) equipped with micromanipulators (Narishige, Tokyo, Japan). The first polar body and MII plate were removed by aspiration with enucleation pipette (i.d. 25 to 30-μm). To ensure the successful removal of the metaphase plate, the enucleated oocyte was exposed to UV light for few seconds following incubation in CR1aa or MK-1 medium that supplemented with 10 μg·mL^{-1} Hoechst 33342 (Calbiochem, San Diego, CA, USA). Only successfully enucleated oocytes were transferred into the fresh manipulation medium and used as recipient cytoplasts.

Nuclear transfer and embryo culture

Transfer, fusion and activation : A single cell suspension prepared by standard trypsinization (Hogan et al., 1986) was aspirated into transfer pipette (i.d. 20 μm) and inserted into the perivitelline space of the recipient cytoplast through the slit made during enucleation. To facilitate close contact between the donor cell and recipient cytoplast, the donor cell and a small volume of cytoplasm were repeatedly aspirated into a pipette until the cell membranes adhered completely to each other. Prior to fusion, the couplets were equilibrated in a mixture of CR1aa or MK-1 medium with Zimmerman fusion medium (2:1; 1:1), then in fusion medium alone. Subsequently, the couplets were transferred to a fusion chamber consisting of two electrodes, 1.0 mm apart, overlaid with the fusion medium. Fusion was induced by a single DC pulse of 1 kv/cm for 50 μs, delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA). After induction of fusion, the couplets were returned to CR1aa or MK1 medium supplemented with 5% FBS. Fusion was determined microscopically 20 min after and 40 min after the onset of incubation. The successfully fused couplets were chemically activated by incubating in 10 μg mL^{-1} calcium ionophore (Sigma) for 5 min followed by culturing in 10 μg mL^{-1} cycloheximide (Wako) dissolved in CR1aa or MK-1 supplemented with 0.3% BSA for 5 h at 38.0°C in 5% CO_2 in an atmosphere of air.

In vitro culture of nuclear transfer embryos : Embryo culture was performed in 100 μL drops (3 to 5 embryos/drop) of CR1aa or MK1 medium supplemented with 5% FBS overlaid with mineral oil at 38.0°C in 5% CO_2 in an atmosphere of air. Embryos cultured in each medium were examined every 24 h following fusion. We recorded the numbers of cleaved embryos on Day 2 and the number that had developed to the morula stage on Day 5 and to blastocysts on Day 7 after fusion.

Statistical analysis

Three to five independent experiments were conducted and results from all replicates were pooled. The data were analyzed statistically by chi-squared analysis or, when some

expected values were less than 0.05, Fisher's exact probability test was used. Differences with a probability value of (p) less than 0.05 were considered significant.

RESULTS

A total 193 of 314 reconstructed embryos (61.5%) successfully fused, and 52% to 73% of these reconstructed embryos underwent cleavage by 48 h after culture. A comparison of the development of embryos reconstructed with cumulus or oviduct cells and cultured in either CR1aa or MK1 media is presented in table 1.

Without regard to the type of donor cells, the development of fused somatic cell–oocyte couplets to the blastocyst stage was not significantly different ($p>0.05$) between cumulus cell and oviduct cells when they were cultured in MK1 or CR1aa media. However, the development of reconstructed embryos in the MK1 group tended to be higher than those in the CR1aa group. The percentage undergoing cleavage and development to the morula stage was significantly higher ($p<0.05$) when reconstructed embryos were cultured in MK1 medium, compared with those cultured CR1aa medium.

When the development of embryos reconstructed with cumulus or oviduct cells was analyzed within the same culture media group (MK1 or CR1aa), no significant differences in the percentage of fusion, cleavage, or development to the morula and blastocyst stages were recorded in the CR1aa group ($p>0.05$). The development of reconstructed embryos in the MK1 group also revealed a similar pattern to that in the CR1aa group: no significant differences were found in the percentage of fusion, cleavage, or development to morula and blastocyst stages ($p>0.05$). These findings suggest that serum-starved cumulus and oviduct cells make a similar contribution to the *in vitro* development of cat reconstructed embryos when cultured in the same medium.

DISCUSSION

The present study implies that nuclei from feline somatic cells are capable of being reprogrammed by the oocyte cytoplasmic environment and obtain the ability to develop to the blastocyst stage after nuclear transfer (fig. 1). To our knowledge, this is the first demonstration of *in vitro* development to the blastocyst stage of reconstructed embryos in feline species. In nuclear transfer, two distinct approaches are thought to increase the efficiency of cloning by nuclear transfer when using mature oocytes as recipient cytoplasts and diploid cells as donor karyoplasts. The first approach is the transfer of diploid cells in G1 or G0 stage to young-matured oocytes; and the second is by transferring cells in G1, S or G2 phase to preactivated oocytes (Campbell et al., 1996, 1999). In the present study, serum-starved somatic cells (cumulus and oviduct cells) were selected as donor cells because they can be easily obtained from ovarihysterectomized cats at veterinary clinics, and because of the recent progress in somatic cell nuclear transfer in farm animals.

The *in vitro* development of cat reconstructed embryos after fusion reflects the enhanced potential of MK1 medium in supporting early development of cat reconstructed embryos compared to CR1aa. Both cumulus and oviduct cells provide blastocyst formation when transferred into enucleated oocytes. However, the overall blastocyst formation the present study was low compared to the

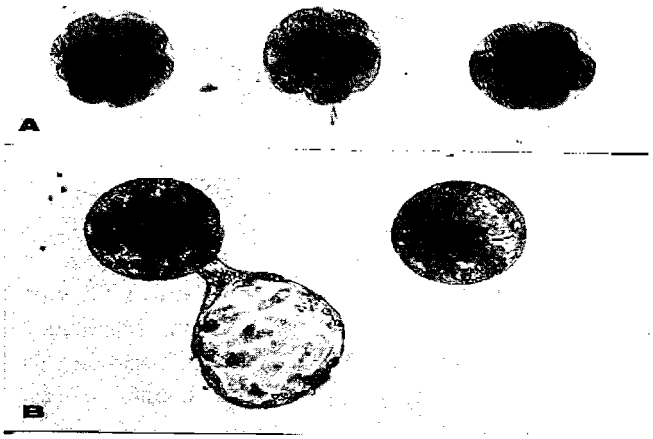

Figure 1. Development *in vitro* of cat embryo reconstructed by somatic cell. (A) Eight- to 16-cell stage embryos at Day 3 after fusion. (B) Blastocyst stage at culture Day 7 after fusion

Table 1. Development of reconstructed embryos derived from cumulus and oviduct cells after culture *in vitro* in MK1 or CR1aa medium

Culture media	Donor cells	No. of reconstructed embryos	No. of fused (%)	Cleavage (%) *	No. developed to *	
					Morula (%)	Blastocyst (%)
MK1	Cumulus	60	39 (65.0)	33 (84.6)[a]	14 (35.9)[a]	6 (15.4)
	Oviduct	99	52 (52.5)	45 (86.5)[a]	23 (44.2)[a]	9 (17.3)
CR1aa	Cumulus	80	58 (72.5)	38 (65.5)[b]	8 (13.8)[b]	5 (8.6)
	Oviduct	75	44 (58.7)	26 (59.0)[b]	6 (13.6)[b]	3 (6.8)

* Based on number of fused couplets. a-b Values with different superscripts within a column differ significantly ($p<0.05$ or less).

development of *in vitro* cat embryo produced by IVF (Kanda et al., 1998) and nuclear transfer in farm animals (Kato et al., 1998). In the present study, some basic requirements of nuclear transfer in domestic cat, such as the sensitivity of cat oocytes to parthenogenetic activation or pulse strength, have not been optimized. Nonetheless, greater percentages of embryos proceeding to cleavage after fusion indicating that cat oocyte can be activated by the combination of a calcium-elevating agent (calcium ionophore) and a protein synthesis inhibitor (cycloheximide), which have been widely used in nuclear transfer in farm animals.

Blocking of development from morula to blastocyst stage has been reported to occur in the cat IVF system (Johnston et al., 1991a, Roth et al., 1994). In the present study, the smaller proportion of reconstructed embryos developing to the blastocyst stage in MK1 (15. 3% and 17.3%) and CR1aa (6.8% and 8.6%) medium indicated that developmental blocks also occurred in cat reconstructed embryos. The developmental block in cat embryo has been reported resistant to numerous alterations in culture conditions, including temperature and gas atmosphere (Johnston et al., 1991b), cell co-culture techniques and protein source (Johnston et al., 1993, Kanda et al., 1998). The components of CR1aa and MK1 media are almost similar; however, each contains a different source of energy substrate (glucose in MK1, and glutamine in CR1aa). Temporal alteration of glucose/glutamine has been reported to influence the *in vitro* development of cat embryos, but the substitution of glutamine for glucose did not significantly enhance the development of IVF-derived cat embryo to blastocyst (Swanson et al., 1996). This explanation may partially reflect the low percentage of blastocyst formation in CR1aa medium. Further study on culture systems, such as temporal medium substitution during culture, will yield valuable information on improving the efficiency of *in vitro* culture of cat reconstructed embryos.

This study may have important implications for conservation of wild cat species in the future, since *in vitro* culture techniques in the domestic cat have been widely used as a model for the development of *in vitro* techniques for other wild cat species. The ability to use recipient cytoplasts of a different species from the nuclear donor could solve some of the difficulties associated with using nuclear transfer to rescue endangered species for which oocyte availability would be limited. A preliminary report on production of blastocysts from closely related species (Meirelles et al., 1999) or interspecies animals (Dominko et al., 1998) demonstrated that donor cells could be reprogrammed by oocytes from closely related species or other species. However, further study is still needed to improve the efficiency of nuclear transfer in the domestic cat before it could be extrapolated to wild cats or other endangered species.

In conclusion, in this study somatic cell nuclear transfer was successfully adopted in the domestic cat and the subsequent reconstructed embryos developed to the blastocyst stage when subjected to further culture. The culture medium has significant impact on the early development of reconstructed cat embryos.

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