In Vitro Development of Interspecies Nuclear Transfer Embryos: Effects of Culture Systems

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ABSTRACT

Porcine fibroblasts were transferred into enucleated bovine oocytes for the interspecies nuclear transfer (NT). After NT, the embryos were cultured in three different culture systems. The media used for the experiment were CR1aa and NCSU23. The culture systems used for the experiment were: 1. Culture in CR1aa for 7 days (CR). 2. Culture in CR1aa for 2 days and subsequently in NCSU23 for 5 days (CR-NC). 3. Culture in NCSU23 for 7 days (NC). Bovine (intraspecies) NT group was used as a control. The oocytes in bovine NT group were treated the same as interspecies NT embryos except using bovine fibroblasts as nuclear donors. Regardless of their nuclear origin (interspecies vs bovine), the embryos in CR (68.4% vs 77.2%) and CR-NC (67.8% vs 70.5%) showed better developmental competence to the 2-cell stage ($p<0.05$) than those in NC (41.0% vs 10.0%). Bovine NT embryos in CR-NC did not develop over the 4-cell stage after the medium replacement, while interspecies NT embryos in CR-NC continued to develop and could reach over the 8-cell stage (12.2%). Blastocysts were only found in bovine NT group (17.4%), but no blastocyst was found in interspecies NT group. This study suggests that the development of interspecies NT embryos mostly depends on their recipient cytoplasm during the culture *in vitro*.

(Key words : Interspecies, Nuclear transfer, *In vitro* culture)

INTRODUCTION

Interspecies nuclear transfer (NT) is a useful tool for the preservation of endangered or extinct animal species because of the limited availability of oocytes from those animals. It has been reported that bovine oocytes can be used as the universal host cytoplast for reprogramming of somatic cell nucleus from various mammalian species (Dominko et al., 1999). Furthermore, a recent study reported that the gaur (*Bos gaurus*) nuclei transferred into bovine oocytes to direct normal fetal development with differentiation could lead to the establishment of complex tissue and organ systems (Lanza et al., 2000).

Each mammalian species has its own culture system for *in vitro* development of preimplantation stage embryos such as KSOM for mice (Lawitts and Biggers, 1993), R1ECM for rats (Miyoshi et al., 1997), NCSU23 for pigs (Petters and Wells, 1993) and CR1aa for cattle (Rosenkrans and First, 1993). Inappropriate culture condition disturbs development of preimplantation embryos. However, little is known about the culture system for interspecies NT embryos although some reports used culture systems for recipient cytoplasts (Dominko et al., 1999; Kitiyanant et al., 2001). Since metabolism of the mammalian embryo is largely controlled by mitochondria in its cytoplasm (Cummins, 1998) the culture system for recipient cytoplast may be appropriate for interspecies NT embryos.

It is well known that mammalian embryos express their own genes after maternal embryonic (zygotic) transition (MET or MZT) and transcripts of maternal origin in the cytoplasm gradually disappeared (Bachvarova et al., 1989; Henrion et al., 1997). It is also reported that majority of porcine and bovine MET occurs at the 4- and 8- cell stages respectively (Prather, 1993, Eyestone and First, 1986) although zygotic transcription initiates at the 2-cell stage in both species (Anderson et al., 2001; Barnes and First, 1991). Interspecies NT embryos at early stage may depend on maternal transcripts which already exist in the cytoplasm for their development. However, gene transcripts from its nucleus may also affect further development of those embryos after MET. Although embryo culture system designed for the recipient cytoplasm may be one of the best options for interspecies NT embryos, embryo culture system for

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the species of nuclear donor cells may support development of interspecies NT embryos after MET.

In this study, interspecies NT embryos which consist of porcine nucleus and bovine cytoplasm were cultured in bovine embryo culture medium (CR1aa) and the medium was replaced with porcine embryo culture medium (NCSU23) at porcine MET point, then *in vitro* development was evaluated.

MATERIALS AND METHODS

Reagents

All inorganic and organic compounds were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise stated.

In Vitro Maturation of Bovine Oocytes

Slaughterhouse ovaries were collected from Holstein cows, placed in saline (30℃), and transported within 2 h to the laboratory. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3 to 6-mm follicles using an 18-gauge needle. COCs were collected into Hepes buffered tissue culture medium 199 (H199; Gibco BRL) supplemented with 0.3% w/v fatty acid free bovine serum albumin. Before *in vitro* maturation, COCs with a compact, non-atretic cumulus oophorus-corona radiata and a homogenous ooplasm were selected. COCs were washed twice in H199 and once in bicarbonate-buffered tissue culture medium 199 (M199; Gibco BRL) supplemented with 10% FBS. Fifty to sixty COCs were transferred in 20 μl of this medium and placed into a 0.5-ml well of maturation medium in 4-well dishes (Nunclon, Denmark) overlaid with paraffin oil. The maturation medium comprised M199 supplemented with 10% FBS, 10 μg/ml follicle stimulating hormone (Antrin, Denka Co., Japan), and 1 μg/ml estradiol. COCs were cultured at 39℃ in a humidified 5% CO_2 in air for 18~20 h. After the maturation, the cumulus cells were completely removed by vortexing COCs in 0.1% hyaluronidase in H199 for 3 min.

Nuclear Donor Cell Preparation

Porcine and bovine fibroblasts were obtained from the primary culture of ear skin biopsies. Tissues were cut into small pieces, and cells were dispersed by exposure to 0.25% (w/v) trypsin solution (Gibco BRL, Grand Island, NY). Cells were cultured in D-MEM (Gibco BRL) supplemented with 10% FBS (Gibco BRL). For each passage, cells were cultured until confluent, were disaggregated by incubation in a 0.25% trypsin solution for 1 min at 37℃, and were allocated to three new dishes for further passaging. The cell lines have been maintained in culture for at least 6 passages. For long-term storage, the cells were collected after trypsinization, frozen in 10% dimethyl sulfoxide, and stored in liquid nitrogen.

Enucleation

Before enucleation, oocytes were incubated in H199 containing 7.5 μg/ml cytochalasin B for 20 min. Zona pellucidae of oocytes matured for 18~20 h were cut with glass needle to make a slit near the first polar boby (PB1). Enucleation was performed with squeezing the oocytes. With this treatment, PB1 and the second metaphase plate in a small volume of surrounding cytoplasm were extruded from the cytoplast. Enucleated cytoplasts were then stained in H199 containing 5 μg/ml Hoechst33342 for 10 min. Enucleation was confirmed by the absence of nucleus under ultraviolet light. After the enucleation, resulting cytoplasts were washed extensively in H199 and were held in this medium until the injection of donor cells.

Preparation of Donor Cells

The cells starved by 0.5% FBS for 4 to 7 days were used for NT. Immediately before injection, cell suspension of the donor cells was prepared by standard trypsinization. The cells were pelleted and re-suspended in H199 and remained in this medium until the NT. Porcine and bovine fibroblasts were used for interspecies and bovine NT, respectively.

Nuclear Transfer

A 30- to 35-μm (external diameter) injection pipette containing the donor cell was introduced through the slit of the zona pellucida as made during enucleation, and the cell was placed between the zona pellucida and the cytoplast membrane to facilitate close membrane contact for subsequent fusion. After the injection, the reconstructed embryos were remained in H199 until fusion.

Cell Fusion

Reconstructed embryos were electrically fused at 24 h post-start of maturation (hpm) in buffer comprising 0.26 M mannitol, 0.1 mM calcium, 0.1 mM magnesium and 0.01% polyvinyl alcohol. Fusion was performed at room temperature, in a chamber with two stainless steel electrodes 3.2 mm apart filled with fusion buffer. The reconstructed embryos were manually aligned with a fine glass needle, so that the contact surface between the cytoplast and the donor cell was parallel to the electrodes. Cell fusion was induced with two DC pulses of 1.8 kV/cm for 15 μs each, 1 s apart, delivered by a BTX Electrocell Manipulator 2001 (BTX). After the electrical stimulus, the reconstructed embryos were washed and incubated in H199 for 30 min, and then

examined for fusion by light microscopy.

Activation

After the fusion, only fused embryos were cultured for a period of 4 h in CR1aa before chemical activation. The activation was induced by an incubation in 20 μl drops of 5 μM ionomycin in HbT for 4 min at 37℃. Embryos were then extensively washed in H199 for 5 min before culture in 1.9 mM 6-dimethylaminopurine (6-DMAP) for 4 h.

Culture of Inter-Species NT Embryos

All the media used here were based on CR1aa (Rosenkrans & First, 1991) and NCSU23 (Petters & Wells, 1993). Embryo culture was performed in 30 μl drops of the medium overlaid with paraffin oil. Whenever possible, groups of seven to ten embryos were cultured together. Reconstructed interspecies NT oocytes were cultured in three different culture systems. The culture systems used for the first experiment were: 1. Culture in CR1aa for 7 days (CR). 2. Culture in CR1aa for 2 days and subsequently in NCSU23 for 5 days (CR-NC). 3. Culture in NCSU23 for 7 days (NC). As control, bovine (intraspecies) NT embryos were used. The oocytes in intra species NT group were treated the same as inter-species NT embryos except using bovine fibroblasts as nuclear donors. All embryos were cultured in a humidified incubator at 39℃ in a 5% CO_2 in air. After *in vitro* culture, all embryos were stained with Hoechst 33342, and the embryonic development was monitored under the fluorescent microscope.

Statistic Analysis

Experiment was repeated four times. Differences in the mean percentages of cleavage and further embryonic development among the treatments were analyzed by Chi-Square test. A value of $p<0.05$ was accepted as an indication of statistical significance.

RESULTS

Regardless of their nuclear origin (interspecies vs bovine, respectively) the embryos in CR (68.4%, 67/98 and 77.2%, 71/92) and CR-NC (67.8%, 61/90 and 70.5%, 62/88) obtained better developmental competence to the 2-cell stage ($p<0.05$) than those in NC (41.0%, 41/100 and 39.4%, 26/66). Bovine NT embryos in CR-NC did not reach the 4-cell stage after the medium replacement. None of those in NC did the 2-cell stage, either. However, 12.2% (11/90) of interspecies NT embryos in CR-NC developed to the 8-cell stage, and 2% (2/100) of those in NC developed to the 4-cell stage. Both in interspecies and bovine NT, the embryos in CR showed higher developmental rates to the 8-cell stage (36.7%, 36/98 and 52.2%, 48/92) than those in the other culture systems ($p<0.05$). Only bovine NT embryos in CR developed to the blastocyst stage (17.4%, 16/92). No blastocysts were obtained from all interspecies NT groups (Fig. 1).

DISCUSSION

As expected, the culture system of bovine embryos (CR) was better for *in vitro* development of interspecies NT embryos than the one of porcine embryos (NC). However, even in this system (CR), interspecies NT embryos were not reached to the blastocyst stage. In contrast, 17.4% of bovine NT embryos were developed to the blastocyst stage when cultured in CR. Dominko et al. (1999) reported *in vitro* production of blastocyst stage embryos after interspecies NT using donor cells from 5 different species including a pig. However, in our system, all embryos were stopped their development before blastocyst stage. These different results may be due to the different source of donor nucleus, because general NT procedure is mostly same between two research groups. Adult porcine ear skin fibroblasts were used as donor cells in this study, while Dominko et al. (1999) used neonate's ear skin fibroblasts for NT. It may be more difficult to reprogram adult ear skin fibroblasts in the bovine oocyte cytoplasm than those from neonates. In addition, regardless of their nuclear origin (interspecies or bovine), most NT embryos were not developed to further stages after their first cleavage when they were cultured in NC system (Fig. 1). To confirm NC system itself, *in vitro* matured and parthenogenetically activated porcine oocytes were cultured in NC for 6 days, and 20~25% of blastocysts were obtained

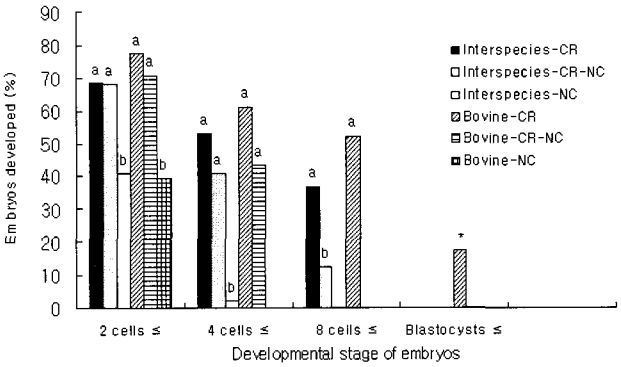

Fig. 1. Development of interspecies nuclear transfer (NT; porcine nucleus / bovine cytoplasm) embryos cultured in three different culture systems ($^{ab}p<0.05$, *Only bovine NT embryos in CR group developed to the blastocyst stage).

consistently (data not shown). The results above indicate that the culture medium for porcine embryonic development may not be suitable for NT embryos reconstructed with bovine enucleated oocytes.

Interestingly, under two-step culture system (CR-NC), some of interspecies NT embryos were developed beyond the 8-cell stage, whereas *in vitro* development of bovine NT embryos was blocked after medium replacement at their 4-cell stage. This result suggests that porcine embryo culture medium may partially support *in vitro* development of interspecies NT embryos after 4-cell stage, although the rate of development beyond the 8-cell stage in CR-NC group was still lower than that in CR group. This time point is largely overlapped with MET of porcine embryos. Hence, reprogrammed porcine nuclei in bovine oocytes passed MET point might express some genes related with early embryonic metabolism, and partially mimic porcine embryos.

This study suggests that the development of interspecies NT embryos primarily depends on their recipient cytoplasm during *in vitro* culture. However, the possibility cannot be excluded that the maternal- embryonic transition of gene expression occurred in early pre implantation stage embryos may play an important role in the development of those embryos.

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