FULL PAPER *Theriogenology*

Production of Blastocysts after Intergeneric Nuclear Transfer of Goral (*Naemorhedus goral*) Somatic Cells into Bovine Oocytes

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ABSTRACT. Interspecies cloning may be a useful method to help conserve endangered species and to study nuclear-cytoplasm interaction. The present study investigated in vitro development of goral (*Naemorhedus goral*) intergeneric nuclear transfer embryos produced by fusing goral fibroblasts with enucleated metaphase II (MII) bovine oocytes. After two to five passages, serum-starved or non-starved goral skin fibroblast cells were transferred into enucleated MII bovine oocytes. Couplets were electrically fused and chemically activated, and then cultured in either modified synthetic oviduct fluid (mSOF) or tissue culture medium-199 (TCM-199) supplemented with 10% FBS. Serum starvation of donor cells did not affect the fusion rate and or development to of cells to the two-cell stage, to more than 9-cells, or to morulae, regardless of culture medium. Three blastocysts from 202 fused embryos were obtained when embryos reconstructed with non- serum- starved donor cells were cultured in mSOF. However, no blastocysts were obtained when the embryos reconstructed with serum-starved donor cells were cultured in mSOF. The total cell number of goral intergeneric embryos averaged 130.3 (range 105–180). In conclusion, this study demonstrated that bovine oocytes can support blastocyst development after intergeneric SCNT with goral fibroblasts.

KEY WORDS: bovine oocytes, goral fibroblasts, intergeneric somatic cell nuclear transfer.

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There is a diversity of opinion regarding the potential of cloning, involving fusion of oocytes with somatic cell nuclei, for conservation of endangered species. Unlike cloning of rodents and domestic animals, because of the scarcity of oocytes, cloning of endangered or extinct species will require the use of an alternative method, such as interspecies somatic cell nuclear transfer (SCNT). Intra-generic SCNT has recently been applied to some endangered species. A gaur (*Bos gaurus*) somatic cell was fused with an enucleated domestic cow (*Bos taurus*) oocyte, and a live offspring was born, although the calf died 2 days later [19]. An endangered argali sheep embryo developed after nuclear transfer with domestic sheep oocytes [20], and using the same approach, a mouflon lamb was born [13]. Like these intra-generic SCNT efforts, there are reports of inter-generic [12] or -spand interspecies [3, 8] preimplantation embryo production with endangered species using SCNT.

The goral is a near-threatened species. Although its population is decreasing, there no reports of attempts to use artificial reproductive technology (ART) for the goral. Therefore, like ART, inter-generic or inter-species SCNT may be one of a potential methods of maintaining the limited goral population and of conserving the species. Accordingly, this study evaluated whether embryonic development could occur following intergeneric SCNT of goral into enucleated bovine oocytes. Bovine oocytes were selected in this study because the goral is a member of the bovidae family. In order to determine whether cell culture conditions for the nuclear donor cells affected embryo development, we investigated the effect of serum starvation of donor cells prior to SCNT. Furthermore, in order to evaluate embryo quality, we determined total cell numbers in the goral intergeneric nuclear- transferred blastocysts.

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MATERIALS AND METHODS

Collection of goral tissue and primary cell culture: A skin biopsy was obtained from an ear of a 10 year-old female goral (*Naemorhedus goral*), housed at the Samsung Everland Zoo in the city of Yong-In, Kyung-Gi Province, Korea. The tissue biopsy was transported to the laboratory at 4°C in phosphate buffered saline (PBS; Life Technologyies, Rockville, MD) supplemented with 0.5% (v/v) penicillin-streptomycin (P/S; Sigma-Aldrich Corp., St. Louis, MO). The tissue was washed several times with PBS and then was minced into small pieces with a surgical blade. The minced pieces were digested in 0.25% trypsin-EDTA (Life Technologies) at 39°C in a humidified atmosphere of 5% CO_2 for 1 to 2 hr. The trypsinized tissue was vortexed and washed at least 3 times in PBS by centrifugation at 300 × g for 3 min. After final washing, Dulbecco's modified Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10% FBS (v/v) was added to the pellet and the cell suspension was placed into a culture dish at 39°C, in 5% CO_2 in humidified air. After removal of unattached clumps of cells or explants, attached cells were fur-

ther cultured for 7 to 8 days until confluent and then subcultured at intervals of 5 to 7 days. For cell stocks, cells were cultured, harvested in the freezing medium, and stored after 2 to 3 passages in liquid nitrogen at –196°C. The freezing medium consisted of 80% (v:v) DMEM, 10% (v:v) DMSO (Sigma-Aldrich Corp.), and 10% (v:v) FBS. Before SCNT, frozen cells were thawed and either maintained in culture medium supplemented with normal serum (10% FBS) or fresh, low serum (1% FBS) culture medium. After 2 to 3 days, the cells were treated with 0.25% trypsin-EDTA at 39°C in a humidified atmosphere of 5% CO_2 for 1 to 2 min and washed with PBS containing 0.5% FBS for SCNT.

In vitro maturation of bovine oocytes: Bovine ovaries collected from a local slaughterhouse were transported to the laboratory within 2 hours in 0.9% (w/v) NaCl solution at 35°. Cumulus-oocyte complexes (COCs) were retrieved from antral follicles 2 to 8 mm in diameter by aspiration with an 18- gauge hypodermic needle attached to a 10 m*l* syringe. The COCs were washed three times in HEPES-buffered tissue culture medium (TCM)-199 supplemented with 10% FBS, 2mM $NaHCO_3$ (Sigma-Aldrich Corp.), 0.5% BSA (Life Technologies), and 1% P/S. The COCs with evenly-granulated cytoplasm and that were enclosed by more than 3 layers of compact cumulus cells were selected. For maturation, a group of 30 to 40 COCs were cultured for 24 hr in bicarbonate-buffered TCM-199 supplemented with 10% (v/v) FBS, 0.005 IU/m*l* follicle-stimulating hormone (FSH, Antrin, Denka Chemical Co., Kanagawa, Japan) and 1 μg/m*l* 17β-estradiol (cat. no. E-8875, Sigma-Aldrich Corp.) at 39°C in a humidified atmosphere of 5% CO_2.

Intergeneric somatic cell nuclear transfer: Aftert 22 hr of maturation culture, expanded cumulus cells of the COCs were removed by repeated pipetting in 0.1% (v/v) hyaluronidase (from the bovine testis, cat. No. H-3884, Sigma-Aldrich Corp.). In HEPES-buffered TCM-199 medium, and oocytes with a first polar body and evenly granulated cytoplasm were selected. These oocytes were enucleated with a micromanipulator (Narishige, Tokyo, Japan) in HEPES-buffered TCM-199 supplemented with 10% (v/v) FBS and 5 μg/m*l* cytochalasin B (cat. no. C-6762, Sigma-Aldrich Corp.). Each oocyte was held with a holding micropipette (120 μm inner diameter), and the zona pellucida was partially dissected with a fine glass needle to create a slit near the first polar body. The first polar body and adjacent cytoplasm, presumably containing the metaphase-II (M II) chromosomes, were extruded by squeezing with the needle. The Ooocytes were then stained with 5 μg/m*l* bisbenzimide (Hoechst 33342, Sigma-Aldrich Corp.) for 5 min and observed under an inverted microscope equipped with an epifluorescence apparatus. Oocytes still containing DNA were excluded. The enucleated oocytes were placed in TCM-199 supplemented with 10% (v/v) FBS and used for SCNT. Cell suspensions of either actively dividing or serum-starved goral fibroblasts were prepared immediately prior to SCNT. A single cell was selected and deposited into the perivitelline space of each enucleated oocyte through the same slit made previously during enucleation. The couplets were subsequently placed in a fusion medium comprising 0.26 M mannitol, 0.1 mM $MgSO_4$, 0.5 mM HEPES, and 0.05% (w/v) BSA, and transferred into a cell fusion chamber with a stainless steel wire electrode (3.2 mm gap; BTX 453, 3.2 mm gap; BTX, , San Diego, CA) after equilibration for 3 min. Fusion was induced by two DC pulses of 1.75 kV/cm for 15 microseconds using a BTX Electro Cell Manipulator 200. Fusion of the donor cell and the ooplast was observed 1 hr after electric stimulation under a stereomicroscope. At 4 hr after fusion, chemical activation was induced by incubating embryos in TCM-199 containing 5 μM ionomycin (Sigma-Aldrich Corp.) for 4 min at 39°C. Reconstructed embryos were then washed thoroughly in ionomycin-free TCM-199 or modified synthetic oviductal fluid (mSOF) and further incubated for 4 hr in TCM-199 or mSOF supplemented with 1.9 mM 6-dimethylaminopurine (6-DMAP, cat. no. D-2629, Sigma-Aldrich Corp.). The formula of mSOF was basically the same as the original formulation [5], except for the concentration of glucose (1.5 mM) and the addition of 2% MEM essential and 1% nonessential amino acids (Life Technologies.), 8 mg/m*l* BSA (fatty acid -free, fFraction V, Sigma-Aldrich Corp.), and 1% (v/v) of a solution containing insulin, transferrintransferring, and sodium seleniumte (ITS, cat. no. I-3146, Sigma-Aldrich Corp.). The osmolarity and pH of the mSOF were 270 to 280 mOsm and 7.2 to 7.3, respectively.

Culture of reconstructed embryos: After 6-DMAP treatment, the reconstructed embryos were cultured in 25 μl microdrops of either TCM-199 or mSOF under mineral oil (Sigma-Aldrich Corp.) in groups of 7 to 10 embryos for 148 hours at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Embryo development to the 2-cell (cleavage), ≥ 9-cell, morula, and blastocyst stages was evaluated at 48 hours, 72 hr, and 148 hr of culture.

Differential staining: Blastocysts were incubated in 500 μl of BSA-free, HEPES-buffered TCM-199 supplemented with 1% (v/v) Triton X-100 and 100 μg/m*l* propidium iodide for 30 sec. When the trophectoderm (TE) cell color visibly changed to red and shrank slightly following treatment, the blastocysts were incubated at 4°C overnight a in 500 μl fixative solution consisting of 25 μg/m*l* bisbenzimide in absolute ethanol. The blastocysts were then placed in 99% (v/v) glycerol, and mounted onto a glass microscope slide in a drop of glycerol solution. Cell numbers were counted using epifluorescence microscopy. Inner cell mass (ICM) nuclei labeled with bisbenzimide appeared blue, and TE labeled with both bisbenzimide and propidium iodide appeared pink.

Statistical analysis: Multiple comparisons (LSD) were implemented using Generalized Linear Models in the SAS 8.12 program. When significance of main effects in each experimental parameter was detected, subsequent comparison was made by the least squares method. Differences among the treatments were considered statistically significant when the P value was less than 0.05.

RESULT

Experiment 1. Embryo development and effect of donor cell treatment and culture media: As shown in Table 1 and Fig. 1, the embryos reconstructed with goral donor cells and enucleated bovine oocytes developed to 2-cells, 8-cells, 16-cells and blastocysts. There were no differences in development when embryos were cultured in TCM-199 or mSOF. Serum starvation of donor cells did not affect the fusion rate of the reconstructed oocytes (75.8%) compared to non-serum starvation (84.3%), nor did it affect cleavage to the 2-cell stage, or development to more than 9-cells, morulae, or blastocysts. Three blastocysts from 202 fused embryos were obtained when the embryos reconstructed with non serum-starved donor cells were cultured in mSOF, and no blastocysts were obstained when the embryos were recon-

Table 1. Effects of donor cell treatment and culture media on development of goral intergeneric embryos reconstructed with nuclear transfer of goral fibroblasts into enucleated bovine oocytes

Donor cell treatment	Fused/total nuclear transferred (%)[a]	Culture medium	Number (%)[b] of embryos developed		
			2-cells	≥ 9-cells	Morulae /blastocysts
Non-starved	200/237 (84.3)	TCM-199	61 (61.0)	27 (27.0)	0 (0.0)
		mSOF	59 (59.0)	34 (34.0)	3 (3.0)*
Serum-starved	204/269 (75.8)	TCM-199	62 (60.8)	44 (42.1)	0 (0)
		mSOF	62 (60.8)	48 (46.1)	0 (0)

Values in the same column are not statistically not different (P>0.05).
*All 3 embryos were blastocysts.
a) Percentage of the total number of oocytes provided for SCNT.
b) Percentage of the total number of oocytes successfully fused.

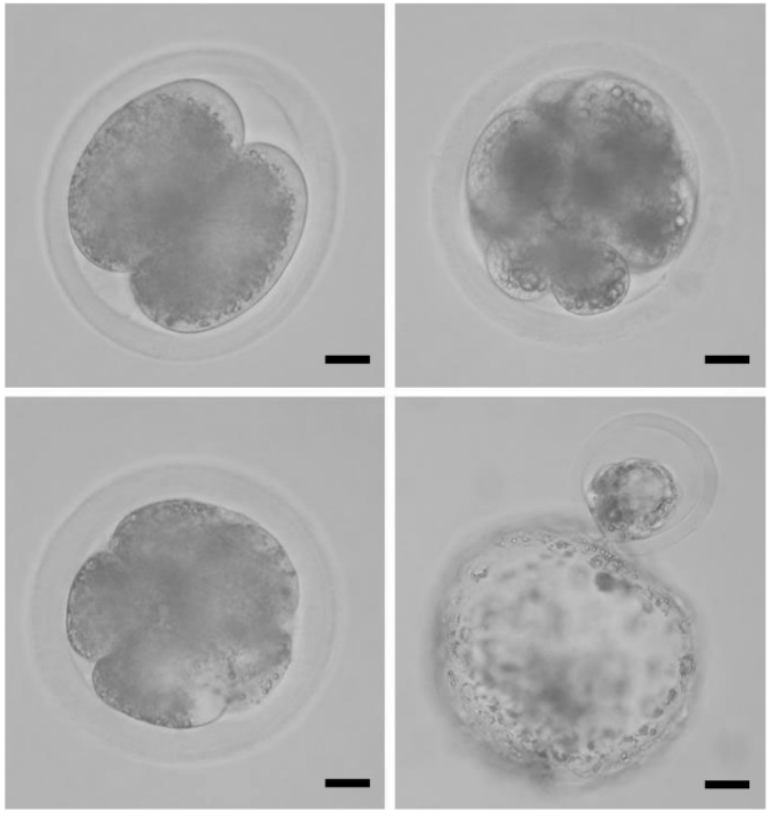

Fig. 1. Preimplantation development of intergeneric embryos derived from somatic cell nuclear transfer of goral skin fibroblast cells into enucleated bovine oocytes matured *in vitro*. Photos of embryos at the (a), 2-cell; , (b), 8-cell;, (c), 16-cell;, and (d), hatching blastocyst stages. Magnification = × 200. Scale bar = 20 μm.

structed with serum-starved donor cells. This difference was not significant.

Experiment 2. Total cell numbers of intergeneric nuclear transferred goral blastocysts: Total cell numbers of the three blastocysts produced in mSOF on Day 8 were 105, 106, and 180 (average 130). ICM numbers were 30, 33, and 62 (average 42), and TE numbers were 75, 73, and 118 (average 90), respectively.

DISCUSSION

In this study, we demonstrated preimplantation embryo development of goral-bovine intergeneric nuclear-transferred embryos from adult goral somatic cells and bovine oocytes. Some of the goral-bovine intergeneric nuclear-transferred embryos developed to the blastocyst stage in vitro, indicating that goral fibroblast nuclei can dedifferentiate in enucleated MII bovine oocytes.

We choose bovine oocyte for the recipient oocytes because the goral is a member of the bovidae family and Dominko *et al.* [7] successfully cultured SCNT embryos reconstructed from mammalian (sheep, pigs, monkeys, and rats) somatic cells and enucleated bovine oocytes.

Although the cell cycle synchrony between donor nucleus and recipient cytoplast is considered to be important for nuclear reprogramming and successful SCNT, there has been debate about the donor nucleus cell cycle stage required for SCNT. Serum starvation adjusts the cell cycle of somatic cells to the G0 phase [4]. Moreover, the production of live offspring from adult and embryonic somatic cell lines was reported to be possible only when G0 phase cells were employed [14, 17]. However, more recent studies have demonstrated that the G0 stage of the cell cycle is not necessary in bovine SCNT [6] and that cell nuclei at the G2/M-stage in reconstructed embryos could develop to the blastocyst stage [13]. Furthermore, cell-cycle synchronization of donor cells by serum starvation is not required for the production of porcine SCNT embryos [9] and intergeneric SCNT [12]. In the present study, goral-bovine intergeneric nuclear-transferred embryos developed to the blastocyst stage when adult goral somatic cells at 80 to 90% confluency without serum treatments were used as donor cells. As in recent reports [9, 12], our results suggest that it is unnecessary to arrest donor cells at the G0/G1 stage with serum starvation in goral intergeneric SCNT. Furthermore, serum starvation induces cell apoptosis [2, 11]. DNA fragmentation is an early characteristic of apoptotic cells and is an irreversible event. Therefore, apoptotic nuclei, even though the morphology of the cells looks normal, may reduce the development of SCNT -reconstructed embryos. Consistent with this idea, no blastocysts were obtained when serum-starved goral cells were used as donor cells, although this outcome was not significantly different from non serum-starved donor cells.

An important factor governing early development of reconstructed embryos is the *in vitro* culture system. In this regard, Yang *et al.* [21] suggested that the early embryo development block was related to embryo species, culture medium, and culture conditions. For *in vitro* production of bovine cloned embryos, many studies have used mSOF or TCM-199 medium supplemented 10% FBS [15, 16, 18]. However, it is unknown whether the culture medium for interspecies reconstructed embryos should be matched to the culture medium for the donor cell or for the recipient oocyte. Dominko *et al.* [7] successfully cultured SCNT embryos reconstructed from mammalian (sheep, pigs, monkeys, and rats) somatic cells and enucleated bovine oocytes using CR1aa medium. In our results, goral intergeneric embryos reconstructed with enucleated bovine oocytes could develop to the blastocyst stage when cultured in mSOF. Our results show that mSOF medium is more suitable than TCM-199 for culturinge of goral intergeneric SCNT embryos. At the first few mitotic divisions, the embryonic genome has little or no transcriptional activity and proteins, and mRNAs derived from the cytoplasm of the oocyte support development. Our results demonstrated that mSOF medium can successfully support embryonic transcription and allow goral intergeneric embryos to develop to the blastocyst stage. However, at this moment, it is not unknown which components of mSOF play a key role in supporting better embryonic development compared with TCM-199. More studies are necessary to elucidate the exact components of the culture medium and to determine which culture medium is better for goral intergeneric SCNT embryos.

In the present study, we examined the numbers of total cells, ICM, and TE cells. The ability of SCNT embryos to develop to term may be restricted to those having a relatively normal ratio of ICM:total cell numbers. However, there are no reports about the total cell and ICM numbers of normal goral blastocysts. In our laboratory, bovine (*Bos taurus*) IVF embryos contain 150 to 180 cells [1] and bovine SCNT embryos contain 110 to 130 cells [10]. Bovine (*Bos taurus*) IVF embryos have an average of 37 ICM and 89 TE cells, and the ratio of ICM: total cell numbers is 0.3 [1]. Bovine SCNT embryos consist of 32 ICM and 99 TE cells, and the ratio of ICM: total cell number is 0.22 [10]. Because the goral is a member of the bovidae family, we estimated that the normal cell numbers of goral blastocysts is in the range of 100 to 150 cells by comparison with other species of cattle. As expected, we obtained an average of 130 total cells in the goral intergeneric blastocysts. The mean numbers of ICM and TE cells were 42 and 90, respectively and the ratio of ICM:total cell numbers was 0.32. Therefore, in comparison with the results of bovine IVF and SCNT, we believe that intergeneric goral blastocysts have normal ICM and TE numbers and a normal ratio of ICM: total cell number

In conclusion, our study demonstrates that bovine oocytes can support development to the blastocyst stage after goral intergeneric nuclear transfer. It also demonstrated that serum starvation is not necessary for donor cell synchronization in goral intergeneric SCNT.

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