Effect of Cell Cycle Stage on the Development of Embryos Produced by Cumulus Cell Nuclear Transfer in Hanwoo (Korean Cattle)

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ABSTRACT : This study was carried out to investigate the effect of activation timing, cell cycle and passage on the development of embryos produced by cumulus cell nuclear transfer in Hanwoo (Korean cattle). Nuclear donor cumulus cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 38.5℃ in a humidified atmosphere of 5% CO_2 in air. The 1~6 passages of serum deprived or actively dividing cumulus cells were isolated and used as donor cells. The in vitro matured oocytes were enucleated and then the isolated donor cells were introduced. One pulse of 180 volts for 15 μs was applied to induce the fusion between karyoplast and cytoplast. The activation was done before or after the fusion. To activate, oocytes were treated with 10 μM calcium ionophore for 5 min immediately followed by 2 mM 6-dimethylaminopurine for 3 h. The nuclear transfer embryos were cultured in 500 μl of modified CRlaa supplemented with 3 mg/ml BSA in four well dish covered with mineral oil. After 3 days culture, culture medium was changed into modified CRlaa medium containing 1.5 mg/ml BSA and 5% FBS for 4 days. The incubation environment was 5% CO_2, 5% O_2, 90% N_2 at 38.5℃. There was no blastocyst formation when the nuclear transfer embryos were activated before the fusion, whereas, 29.9% of blastocyst formation was shown when the nuclear transfer embryos were activated after the fusion. When serum deprived and actively dividing cumulus cells were used as nuclear donor cells, the developmental rates to blastocyst were 38.5% and 40.6%, respectively. There was no significant difference between serum deprived and actively dividing cells in the developmental rates. The developmental rates to blastocyst according to 1~6 passages were 37.5~44.4%. However, there were no significant differences among passages. These results indicate that 1~6 passage cumulus cell irrespective of cell cycle could support development of nuclear transfer embryos activated after the fusion. (*Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 6 : 759-764*)

Key Words : Cell Cycle, Nuclear Transfer, Donor Cumulus Cell, Blastocyst

INTRODUCTION

The possibility of generating genetically identical offspring in livestock has evolved considerably during last two decades from the first embryo splitting experiments (Willadsen, 1979) up to the recent result obtained after the Dolly break-through achieved with somatic cell cloning (Wilmut et al., 1997). Since then, cows (Wells et al., 1999) and mice (Wakayama et al., 1998) have been cloned from adult cell donors, and their cloned offspring have proved fertile (Wakayama et al., 1998).

In nuclear transfer, the transferred donor nucleus underwent nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC). The PCC was induced by high levels of maturation promoting factor (MPF) and resulted in chromosome damage (Campbell et al., 1993). Also, the degree of PCC was affected by the cell cycle stages of donor nuclei. Collas et al. (1992) reported that the development of rabbit nuclear transplant embryos to blastocysts was greater when blastomeres in G1 or early S stage were transferred than when nuclei were G2 or late S stage. For cattle, however, blastomeres after 2 cell stage were mostly S stage, and G1 stage was very short or absent, and thus synchronization of bovine blastomeres into G1 stage was very difficult or impossible (Barnes and Eystone, 1990; Barnes et al., 1993).

In nuclear transfer using blastomere, two methods have been developed to synchronize cell cycle between donor blastomere and recipient cytoplasm; the first is to induce the donor cells into G1/S stage by treatment with colcemid or nocodazole (Cheong et al., 1993; Techakumpu et al., 1993; Campbell et al., 1994), and the second is to synchronize recipient cytoplasm into the S stage by the activation agents (Barnes et al., 1993; Aoyagi et al., 1994; Kono et al., 1994). The latter method is now widely used. Accordingly, the most important factor is probably cell cycle synchronization for the production of blastocysts by the nuclear transfer.

In somatic cell nuclear transfer, the donor cells were induced to exit the growth cycle and enter into G0 stage (Wilmut et al., 1997). For cattle, Kato et al. (1998) reported the production of cloned calves derived from G0 induced oviduct and cumulus cells. However, Cibelli et al. (1998) reported the generation of cloned transgenic calves from actively dividing fetal fibroblast, and suggested that quiescent state of the donor cell could not support the full developmental capacity of embryos with transferred fetal or adult somatic cells.

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During the past decades, Hanwoo of Korean native cattle have been improved by artificial insemination. Nowadays, as mentioned above, nuclear transfer of adult somatic cells from farm animals is the most efficient technique for obtaining large numbers of genetically identical and superior animals. Especially, cumulus cells are appropriate for cloning females, because they can be easily obtained without injury to the animals. So this study was carried out to investigate the effect of activation timing, donor cell cycle and donor cell passage on the developmental ability of cumulus cell nuclear transfer embryos in Hanwoo.

MATERIALS AND METHODS

Oocyte collection and maturation

Ovaries were collected from the local slaughter house and transported in 25~30℃ saline in a thermos to the laboratory. Ovaries were washed three times with the saline and stored until aspiration. Cumulus-oocyte complexes (COCs) were recoverd by aspiration of 2~6 mm follicles using a 10 ml syringe with 18 gauge needle and stored in Dulbecco's phosphate buffered saline (D-PBS, GIBCO, USA) supplemented with 5% fetal bovine serum (FBS, GIBCO, USA). The aspirated follicular fluid was put in a 60 mm petri dish (Falcon, USA) and only the COCs surrounded with at least three or four compact layers of cumulus cells and with evenly distributed cytoplasm were selected for in vitro maturation under a stereo-microscope (×40).

The maturation medium was tissue culture medium 199 (TCM199, GIBCO, USA) supplemented with 10% FBS and 1% antibiotic-antimycotic solution (GIBCO, USA). The COCs were washed three times with maturation medium and then cultured for 20~22 h in preincubated 500 μl drop of maturation medium in a 35 mm petri dish (Falcon, USA) covered with mineral oil (E. R. Squib Sons, Inc., USA). The incubation environment was 5% CO_2 and 95% humidified air at 38.5℃.

Nuclear transfer procedure

Enucleation : Cumulus cells were removed from the oocytes matured for 18~20 h by vortexing in calcium and magnesium free PBS supplemented with 0.1% hyaluronidase (SIGMA, USA) for 5 min. Only oocytes with the first polar body were selected as recipient oocytes. All micromanipulations were carried out at room temperature using a Narishige micromanipulator and inverted microscope (Nikon, Japan). The recipient oocytes were placed in a 30 μl drop of TCM199 supplemented with 20% FBS and 50 μg/ml phytohemaglutinin (PHA, SIGMA, USA). An oocyte was held by holding pipette (outer diameter was approximately 90~120 μm). Enucleation of oocytes was carried out by cutting the part of the zona pellucida near the first polar body, and then a small volume of cytoplasm surrounding the first polar body was squeezed out through the slit made at enucleation with a cutting needle. After manipulation, oocytes were stained with 2 μg/ml 33342 Hoechst dye (SIGMA, USA) and observed for a few seconds by fluorescent microscopy to select successfully enucleated oocytes. Enucleated oocytes were transferred to TCM199 supplemented with 20% FBS and washed three times.

Donor cumulus cell preparation : After the COCs were collected by the same method as that of *in vitro* maturation, the COCs were washed three times in calcium and magnesium free PBS supplemented with 5% FBS by centrifugation at 1,200 rpm for 5 min and then resuspended with Dulbecco's modified Eagle medium (D-MEM, GIBCO, USA) supplemented with 10% FBS and 1% antibiotic-antimycotic solution. The suspended cumulus cells were plated in 100 mm petri dish (Falcon, USA) and cultured at an atmosphere of 5% CO_2 in 95% humidified air at 38.5℃.

After 24 h culture, the cumulus cells were washed by replacing the culture medium. The culture medium was replaced with fresh medium at every 48~72 h. The attached cumulus cells were passaged by the trypsinization, or cryopreserved when the culture became confluent. Single cell isolation was obtained by incubation in 0.5% trypsin (SIGMA, USA) solution for 5 min. Cell cycle parameters were determined by monitoring DNA content of cumulus cell nuclei, collected under rigorously controlled conditions, by flow cytometry (Schuetz et al., 1996).

Inducement of quiescent state : After 3 or 4 passages, cells were washed with calcium and magnesium free PBS supplemented with 0.5% FBS. Then, cells were incubated in D-MEM supplemented with 0.5% FBS at an atmosphere of 5% CO_2 in air at 38.5℃ for 4 or 5 days.

Nuclear transfer : Enucleated oocytes were placed in a 30 μl drop of the same medium as that of enucleation, and donor cells were placed in a 20 μl drop of calcium and magnesium free PBS supplemented with 5% FBS. Donor cells were introduced into the perivitelline space of the recipient oocytes through the hole made at enucleation by an injection pipette (inner diameter was approximately 10~15 μm). The nuclear transferred oocytes were washed three times in TCM199 supplemented with 20% FBS.

Fusion : Fusion between karyoplast and cytoplast was accomplished by the use of electric pulse. The karyoplast and cytoplast complexes were placed in the Zimmermann's fusion medium for equilibration and then placed between the 1 mm gap of an electrofusion

chamber (PN 450-1) filled with the fusion medium. The karyoplast and cytoplast complexes were aligned manually with mouth pipette and then electric current was applied by an Electro Cell Manipulator ECM® (BTX Inc, USA). One pulse of 180 volts for 15 μs was applied to induce the fusion between karyoplast and cytoplast. The karyoplast-cytoplast complexes were washed in TCM199 supplemented with 20% FBS, and the fusion was evaluated by microscopic examination at 30~60 min after fusion treatment.

Activation of embryos produced by nuclear transfer : To activate the fused embryos, the embryos were treated with 10 μM calcium ionophore (Ca, SIGMA, USA) for 5 min immediately followed by 2 mM 6-dimethylaminopurine (DMAP, SIGMA, USA) for 3 h.

In vitro culture

After the activation treatment, fused embryos were washed three times with culture medium and then cultured on the cumulus cell feeder layer of 1~1.2×10^5/ml in 500 μl of modified CRlaa supplemented with 3 mg/ml BSA in a four well dish (Nunclon, USA) covered with mineral oil. Co-culture cumulus cells were prepared by the treatment of a confluent cumulus cell culture with 10 μg/ml mitomycin C for 2.5 h. The culture media were changed according to experiment; details were described in the experimental design. The incubation environment was 5% CO_2 in 95% humidified air or 5% CO_2, 5% O_2, 90% N_2 at 38.5℃.

Experimental design

Experiment 1 was designed to investigate the effect of activation timing on the development of embryos produced by cumulus cell nuclear transfer. In treatment 1, the enucleated oocytes were activated at 4 h before the fusion treatment. In treatment 2, the fused embryos were activated at 1 h after the fusion. Actively dividing cells were used as nuclear donors at passages 3~4.

Experiment 2 was designed to investigate the donor cell cycle stage on the development of embryos produced by somatic cell nuclear transfer. In this experiment, actively dividing and serum deprived (G0/G1) cells of cumulus were used as donor cells at passages 3~4. The fused embryos were activated at 1 h after the fusion.

Experiment 3 was designed to investigate the effect of donor cell passage on the development of embryos produced by cumulus cell nuclear transfer. In this experiment, 1, 2, 3, 4, 5 and 6 passage cells were used. The fused embryos with actively dividing cells were activated at 1 h after the fusion.

The experiment 1, 2 and 3 were repeated three times, respectively.

Statistical analysis

Analyses of variance (ANOVA) were carried out using the SAS package (SAS, 1988) in a completely randomized design. Student's t-test was used to compare mean values of individual treatments when the F-value was significant ($p<0.05$).

RESULTS

Activation timing

As shown in table 1, fusion rate in preactivation and delayed activation was 43.2 and 66.3% ($p<0.05$), respectively. Even though there was no blastocyst formation when the enucleated oocytes were activated at 4 h before the fusion (preactivation), 29.9% of blastocyst was produced when the fused oocytes were activated at 1 h after the fusion (delayed activation). These results indicate that delayed activation may be optimal for the development of embryos produced by cumulus cell nuclear transfer.

Donor cell cycle stage

This experiment was conducted to investigate the effect of donor cell stages on the development of embryos produced cumulus cell nuclear transfer. Serum deprived or actively dividing cumulus cells were used as nuclear donor cells at passages 3~4. As shown in table 2, there was no significant difference for developmental rates between serum deprived and

Table 1. Effect of the activation timing on fusion rate and the development of embryos produced by cumulus cell nuclear transfer in Hanwoo

Activation timing[1]	No. of replicates	No. of oocytes enucleated	No. (%) of oocytes fused	No. (%) of oocytes developed to	
				2 cell	Blastocyst
Preactivation	3	74	32 (43.2)[b]	27 (84.4)	0 (0.0)[b]
Delayed activation	3	101	67 (66.3)[a]	57 (85.1)	20 (29.9)[a]

[a,b] Means with different superscripts within the same columns were significantly different ($P<0.05$).

[1] Preactivation : Enucleated oocytes were activated at 4 h before the fusion and Delayed activation : the fused oocytes were activated at 1 h after the fusion.

actively dividing cumulus cells.

Donor cell passage

This experiment was carried out to investigate the effect of passage on the development of embryos produced by cumulus cell nuclear transfer. As shown in table 3, the range of the cleavage rates to 2 cell by each passage was 71.1~84.8%. The range of the developmental rates to blastocyst was 37.5~44.4%. There were no significant differences among passages in the fusion and developmental rates.

DISCUSSION

Activation timing

To maintain correct ploidy in the nuclear transfer embryos, it is necessary to synchronize cell cycle between cytoplast and karyoplast. Exposure of the donor chromatin to recipient cytoplasm, in which MPF level is high, has been considered as essential for nuclear remodeling (Fulka et al., 1998b). NEBD and PCC are essential procedures for occurrence of nuclear remodeling. When MIIoocytes are used as cytoplast, cell cycle synchronization by the activation is possible. Three types of cytoplast can be available. The first, MIIoocytes are activated concurrently with the nuclear transfer resulting in a transient exposure of donor chromatin; the second, MIIoocytes are activated after the fusion, namely exposure of donor chromatin to a high level of MPF activity (delayed activation); and the third, MIIoocytes are activated before the fusion (preactivation).

In this study, blastocysts were produced by the delayed activation. This was in accordance with the reports that offspring were produced only by the delayed activation for cattle and mouse, but not consistent with the reports that lambs were produced by all these three methods for sheep (Campbell et al., 1996; Cibelli et al., 1998; Wakayama et al., 1998; Wells et al., 1997, 1999). These disagreements among researchers were due to species differences. The rate of decay of MPF kinase activity varied between species (Campbell, 1999).

The oocytes activated by the same electrical pulse used to induced fusion or after injection of the donor cell, the degree and duration of PCC observed were dependent on the timing of exposure, level and rate of decay of kinase activity (Ritchie and Campbell, 1995). These results suggested that the delayed activation and the prolonged exposure of donor chromatin to recipient cytoplasm in which MPF level was high, could support development of nuclear transfer embryos.

Donor cell cycle stage

The cell cycle of donor nucleus transferred to the second metaphase oocytes, in which MPF level is high, affect the chromatin (Campbell et al., 1996). After transfer, G1/G0 nuclei condense as single chromatids but G2 nuclei condense with two chromatids. In these cases, PCC does not cause

Table 2. Effect of donor cell cycle on the development of embryos produced by cumulus cell nuclear transfer in Hanwoo

Cell stage	No. of replicates	No. of oocytes enucleated	No. (%) of oocytes fused[2]	No. (%) of oocytes developed to	
				2 cell	Blastocyst
Serum deprived[1]	3	121	78 (64.5)	66 (84.6)	30 (38.5)
Actively dividing	3	113	64 (56.6)	53 (82.8)	26 (40.6)

[1] Serum deprived cell stage was induced by serum starvation for 4~5 days with DMEM containing 0.5% FCS.
[2] The fused oocytes were activated at 1 h after the fusion.

Table 3. Effect of cumulus cell passage on the development of embryos produced by cumulus cell nuclear transfer in Hanwoo

Passage[1]	No. of replicates	No. (%) of oocytes enucleated	No. (%) of oocytes fused[2]	No. (%) of oocytes developed to	
				2 cell	Blastocyst
1	3	85	52 (61.2)	44 (84.6)	21 (40.4)
2	3	65	36 (55.4)	30 (83.3)	16 (44.4)
3	3	103	65 (63.1)	51 (78.5)	25 (38.5)
4	3	146	92 (63.0)	78 (84.8)	36 (39.1)
5	3	123	64 (52.0)	53 (82.8)	24 (37.5)
6	3	77	45 (58.4)	32 (71.1)	17 (37.8)

[1] Passage was undertaken by the treatment of 0.5% trypsin solution for 5 min.
[2] The fused oocytes with actively dividing cells were activated at 1 h after the fusion.

genetic damage. In contrast, transfer of S nuclei causes genetic damage. The chromatin decondenses and nuclear envelope reforms after the decline of MPF. In this situation, all nuclei regardless of their cell cycle stage then undergo DNA replication. However, if the recipient cytoplasts are activated and MPF kinase activity has declined prior to transfer of donor nucleus, then no NEBD or PCC occur, and DNA synthesis occurs in relation to the cell cycle stage of donor nucleus at the time of transfer. Therefore, cell cycle synchronization is very important for the maintenance of correct ploidy (Campbell et al., 1996; Fulka et al., 1998a; Campbell, 1999). Dolly was generated using G0 induced mammary gland cell (Wilmut et al., 1997). Since then, G0 induced donor cells have been commonly used to produce clones in bovines and goats.

In this study, there was no significant difference for developmental rates between serum deprived and actively dividing cumulus cells. This result was in accordance with the reports that the cloned calves were generated by somatic cell nuclear transfer using actively dividing fetal fibroblast cells (Cibelli et al., 1998; Shiga et al., 1999; Vignon et al., 1999). Also this was consistent with the report of Cibelli et al. (1998) who produced calves by activating nuclear transfer embryos with Ca and DMAP. Calcium increase in the cytoplasm is sufficient to resume the meiosis and extrude the second polar body, but not pronuclear formation. Additional DMAP treatment suppresses reduction division and drives nuclear transfer embryos into interphase as diploid (Susko-Parrish et al., 1994; Fulka et al., 1998b). This function of DMAP might be able to illustrate this result. According to the report of Schuetz et al. (1996), approximately 90% of ovulated mouse cumulus cells was in G1/G0 or S, but G2 phase was less than 10%. Accordingly, the cultured cumulus cell at confluent stage could be used as donor cells without the induction of quiescence (Prather et al., 1999).

Consequently, serum deprived state should not be a prerequisite in somatic cell nuclear transfer.

Donor cell passge

In this study, there was no significant difference among passages when the 1~6 passages of cumulus cells were used in nuclear transfer. Unlike this result, Campbell et al. (1996) reported that live lambs were generated from only 1~3 passage cells without cell cycle synchronization, but no lambs were produced from 6~13 passages unless cell cycle was synchronized. Wilmut et al. (1997) produced Dolly using G0 induced 3 to 6 passage fetal fibroblast cells. Cibelli et al. (1998) used fibroblast cells after 2 passage and produced calves. Zakhartchenko et al. (1999) produced blastocyst using 1 to 5 passage mammary gland cells and ear cells. Vignon et al. (1998) also generated calves using the 3 to 9 passage somatic cells. Consequently, if the cumulus cells show normal growth, there would be no difference among passages in developmental rate.

In conclusion, 1~6 passage cumulus cells irrespective of cell cycle could support blastocyst development of nuclear transfer embryos activated after the fusion.

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