Effect of Protein Supplementation, O_2 Concentration and Co-Culture on the Development of Embryos Produced by Nuclear Transfer Using Cultured Cumulus Cells in Hanwoo (Korean Cattle)

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ABSTRACT : The effect of protein supplementation, O_2 concentration and co-culture on the development of embryos produced by nuclear transfer using cultured cumulus cell was investigated. Recipient oocytes and cumulus cells were obtained from the ovaries of the slaughtered Hanwoo cows. Donor cumulus cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 5% CO_2 in air at 38.5°C. The 1 to 6 passages of 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 15 μs pulse of 180 volts was applied to induce the fusion between karyoplast and cytoplast. The fused embryos were activated with 10 μM calcium ionophore for 5 min and 2 mM 6-dimethylaminopurine for 3 h. To examine the effect of protein supplementation, nuclear transfer (NT) embryos were cultured in one of the following 4 treatments : 1) CR1aa + 3 mg/ml BSA for 7 days ; 2) CR1aa + 10% FBS for 7 days ; 3) CR1aa + 1.5 mg/ml BSA + 5% FBS for 7 days ; and 4) CR1aa + 3 mg/ml BSA for first 3 days and then CR1aa + 1.5 mg/ml BSA + 5% FBS for 4 days. Culture took place at 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. Although there were no significant differences in cleavage rate among different protein supplements, the rates of blastocyst formation were significantly different. When NT embryos were cultured in the medium supplemented with only BSA, they could develop to only morula not to blastocyst. However, when FBS was supplemented, NT embryos developed to blastocyst stage. In order to investigate the effect of O_2 concentration and co-culture, NT embryos were cultured in CR1aa + 1.5 mg/ml BSA + 5% FBS with or without cumulus cell co-culture at an atmosphere of 5% CO_2 in air (20% O_2) or 5% CO_2, 5% O_2, 90% N_2 (5% O_2) at 38.5°C for 7 days. The percentage of blastocyst development was significantly higher when the NT embryos were cultured at an atmosphere of 5% O_2 than that of 20% O_2 ($p<0.05$). However, there was no significant difference between with and without cumulus cell co-culture at an atmosphere of 5% O_2 or 20% O_2. Fifty embryos were transferred to 25 recipients and 5 recipients were pregnant at 100 days. From 5 pregnant cows, only one cow was delivered of female twin. In conclusion, the embryos reconstructed by enucleation of metaphase II oocytes and introduction of the cycling and quiescent cumulus donor cells in Hanwoo had developmental potential to term after embryo transfer to recipient cows. ***(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 9 : 1260-1266)***

Key Words : O_2 Concentration, Blastocyst, Cumulus Cell, Nuclear Transfer, Embryo

INTRODUCTION

The first cloned sheep (Willadsen, 1986) was generated from the culture in an agar cylinder which was transferred to the recipients, but before the *in vivo* culture, embryos were cultured *in vitro*. For cattle, nuclear transfer calves were also born by this method (Prather et al., 1987). Bondioli et al. (1990) also reported that the development to the morula and blastocyst of the nuclear transfer embryos was higher in the ligated sheep oviduct than in vitro. However, Sims et al. (1991) reported that the development to the morula and blastocyst of nuclear transfer embryos increased to 32.6% when they were cultured *in vitro* using CR1aa medium; these very encouraging results and presented a new possibility of nuclear transfer embryo culture *in vitro*.

Kato et al. (1998) also obtained 48.6% (cumulus cell) and 22.7% (fetal fibroblast cell) blastocysts by the culture in CR1aa medium supplemented with BSA and fetal bovine serum (FBS). The medium used for embryo culture was usually supplemented with serum as a protein source. Serum was beneficial for the development of bovine embryos when present in the culture medium from 72 h post insemination, but it could contain a factor that blocks development of embryos when included in the culture medium before the first cleavage division (Pinyopummintr and Bavister, 1994). There was no specific reason to use serum or other protein sources in the culture medium for supporting development of preimplantation embryos, at least up to the morula stage. Serum could stimulate blastocyst development in embryos of livestock species and primates (Bavister, 1995). Similarly, Wang et al. (1997) reported that a greater proportion of cleaved oocytes developed to blastocyst and hatched blastocyst in a medium supplemented with FBS during post cleavage stage. Baguisi et al. (1999) reported that 35-56% blastocysts were obtained when embryos produced by goat somatic cell nuclear transfer were cultured in TCM 199 supplemented with 10% FBS.

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Received January 31, 2001; accepted April 27, 2001

Generally, atmospheric O_2 concentration is approximately 21%, but in the female reproductive tract O_2 concentration it is only 1.5-8.7%. Preimplantation embryos develop in uterus under very low O_2 concentration of 3.5-5.3% (Fischer and Bavister, 1993). Fukui et al. (1991) reported that in medium TCM 199, when IVM/IVF bovine oocytes were co-cultured with bovine oviductal epithelial cells, more development to blastocyst was observed at 5% CO_2 in air than 5% CO_2, 5% O_2, 90% N_2, whereas in medium SOF, 5% CO_2, 5% O_2, 90% N_2 without co-culture supported more development to blastocyst. Nagao et al. (1994) also reported that bovine IVM/IVF embryos could develop to blastocyst stage in a protein free medium without co-culture cells at 5% O_2 concentration. For the culture of embryos with transferred somatic cell, reconstructed embryos were co-cultured in the medium supplemented with the serum or albumin at 5% CO_2 in air (Cibelli et al., 1998 ; Kato et al., 1998 ; Vignon et al., 1998).

Hanwoo was very important native cattle in Korea for use as a draft animal during the past centuries. However, Hanwoo has been improved by artificial insemination for meat production during the past decades. Nowadays, nuclear transfer of adult somatic cells from farm animals is the most efficient technique for obtaining large numbers of genetically identical and superior animals. Therefore, this study was carried out to investigate the effect of protein supplementation, O_2 concentration and co-culture on the development of embryos produced by nuclear transfer using cultured cumulus cell in Hanwoo.

MATERIALS AND METHODS

Experimental design

Experiment 1 was designed to investigate the effect of protein supplementation on the development of embryos produced by cumulus cell nuclear transfer. The fused oocytes were cultured in a 500 μl drop of CR1aa supplemented with 3 mg/ml BSA (Treatment 1), 10% FBS (Treatment 2) and 1.5 mg/ml BSA and 5% FBS (Treatment 3) for 7 days after fusion. In treatment 4, the embryos were cultured in CR1aa supplemented with 3 mg/ml BSA for 3 days, and CR1aa supplemented with 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°C.

Experiment 2 was designed to investigate the effect of O_2 concentration and co-culture on the development of embryos produced by cumulus cell nuclear transfer. The fused embryos were cultured in CR1aa medium + 1.5 mg/ml BSA + 5% FBS with or without cumulus cell co-culture at 5% O_2 (5% CO_2 and 90% N_2) or 20% O_2 (5% CO_2 in air) at 38.5°C for 7 days. The number of replicates in both experiments 1 and 2 was four.

Oocyte collection and maturation

Ovaries were collected from the local slaughter house and transported in 25-30°C saline in a thermos to the laboratory. Ovaries were washed three times with the saline and stored at 37°C until aspiration. Cumulus-oocyte complexes (COCs) were recovered 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 (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 (TCM 199, 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°C.

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. The rates of oocyte maturation with the first polar body were 90-95%. The 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 TCM 199 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 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 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 TCM 199 supplemented with 20% FBS and washed three times.

Donor cell preparation

After the COCs were collected by the same method as

Table 1. Effect of protein supplements on the development of embryos produced by cumulus cell nuclear transfer in Hanwoo

Protein source[1]	No. of oocytes enucleated	No. of oocytes fused	No. (%) of oocytes developed to		
			2 cell	Morula	Blastocyst
T1	91	50 (54.9)[ab]	42 (84.0)	23 (46.0)[a]	0 (0.0)[b]
T2	107	49 (45.8)[b]	39 (79.6)	4 (8.2)[b]	15 (30.6)[a]
T3	105	74 (70.5)[a]	65 (87.8)	5 (6.7)[b]	29 (39.2)[a]
T4	95	53 (55.8)[ab]	44 (83.0)	0 (0.0)[b]	23 (43.4)[a]

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

[1] The nuclear transfer embryos were cultured in CR1aa medium supplemented with 3 mg/ml BSA (T1), 10% FBS (T2), and 1.5 mg/ml BSA and 5% FBS (T3) for 7 days after fusion, respectively. In T4, the embryos were cultured in the medium supplemented with 3 mg/ml BSA for 3 days , and 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°C. Four replicates.

the oocyte collection and 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 placed in 100 mm petri dish (Falcon, USA) and cultured at an atmosphere of 5% CO_2 in 95% humidified air at 38.5°C. After 24 h culture, the cumulus cells were washed by replacing the culture medium. The attached cumulus cells were passaged by the trypsinization or cryopreserved when the culture became confluent. Single cell isolation was obtained by the 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).

Nuclear transfer

Enucleated oocytes were placed in a 30 μl drop of the same medium as the 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 the injection pipette (inner diameter was approximately 10-15 μm). The nuclear transferred oocytes were washed three times in TCM 199 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 fusion medium for equilibration and then placed between the 1 mm gap of an electrofusion chamber (PN 450-1) filled with Zimmerman cell fusion medium (Wolfe and Kraemer, 1992), containing 0.28 M sucrose, 1.0 mM K_2HPO_4, 0.1 mM glutathione, and 0.01 mg/ml BSA ; the concentrations of Mg $(C_2H_3O_2)\cdot 4H_2O$ and Ca $(C_2H_3O_2)_2$ were reduced to 0.05 mM and 0.01 mM, respectively. 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). After the fusion treatment, the karyoplast-cytoplast complexes were washed in TCM 199 supplemented with 20% FBS, and the fusion was evaluated by microscopic examination 30-60 min after fusion treatment.

Activation

The fused embryos were activated in CR1aa + 1.5 mg/ml BSA + 5.0% FBS supplemented with 10 μM calcium ionophore for 5 min immediately followed by 2 mM 6-dimethylaminopurine for 3 h.

***In vitro* culture**

After the activation treatment, fused embryos were washed three times with the culture medium and then cultured on the cumulus cell feeder layer of 1-1.2 × 10^5/ml in 500 μl well of CR1aa supplemented with 1.5mg/ml BSA and 5% FBS in the 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 and 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°C.

Embryo transfer and pregnancy diagnosis

Recipient cows were raised in the Daekwanryeong Branch of National Livestock Research Institute. After 7 days culture of embryos produced by nuclear transfer in Suwon, the National Livestock Research Institute, blastocysts were transported from the institute to the branch in TCM 199 supplemented with 20% FBS. The blastocysts were loaded into 0.25 ml straws using embryo transfer freezing medium (ETFM, GIBCO, USA) supplemented

with 20% FBS and then non-surgically transferred to recipient cows using cassou gun on day 7 after natural estrus. Pregnancy was diagnosed by rectal palpation at day 100.

Statistical analysis

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

RESULTS

The development of the nuclear transfer embryos cultured in CR1aa supplemented with different protein supplements is shown in table 1. Although 46.0% of morula was produced when the embryos were cultured in the medium supplemented with only BSA, there was no blastocyst formation. However, when FBS was supplemented to the medium alone or with BSA, the range of the development to blastocyst of embryos was 30.6-43.4%. This result suggested that FBS showed beneficial effect for blastocyst formation.

As shown in table 2, the development of embryos in subtotal means to 2 cell of nuclear transfer embryos between 5% O_2 and 20% O_2 groups was not significantly different (81.2 vs 71.4), but the development of embryos to blastocyst were significantly higher at 5% O_2 than 20% O_2. There was no significant difference between the coculture and non-coculture groups on the development of embryos to blastocyst at 5% O_2 and 20% O_2, respectively. As shown in table 3, of Hanwoo cows with transferred blastocysts produced by cumulus cell nuclear transfer 20.0% were pregnant at 100 days of gestation. Of five pregnant cows, fetuses of three cows were aborted at 152, 229 and 258 days of gestation, respectively, but two cows were delivered of calves. One cow was delivered of twin and the other cow was delivered of a calf, but the latter calf died immediately after birth. Average birth weight of female twin calves was 40 kg.

DISCUSSION

The results in table 1 showed that embryos cultured in CR1aa supplemented with only BSA at 5% O_2 did not develop to blastocyst stage. However, the embryos cultured in CR1aa supplemented with FBS alone or FBS + BSA developed to blastocyst. The development to blastocyst was higher when FBS was added at post cleavage stage. This result was consistent with the result of Wang et al. (1997) who reported that a greater proportion of embryos was stopped at the morula stage in the medium supplemented with only BSA during post cleavage stage. Development to the blastocyst stage and the hatching rate were better in FBS-supplemented media than in media supplemented with BSA.

However, phasic response of serum on embryo development has been reported (Pinyopummintr and Bavister, 1994) due to its extracellular matrix components like fibronectin or growth factors advantageous to bovine blastocyst development (Larson et al., 1990, 1992a,b). Different types and sources of protein supplementation used in culture media can greatly influence development in vitro, ranging from marked stimulatory to completely inhibitory effects (Kane, 1983). In this study, the development of embryos cultured in CR1aa supplemented with only FBS had biphasic effects and FBS might be essential at post cleavage stage for blastocyst formation of embryos produced by cumulus cell nuclear transfer.

Until recently, somatic cell nuclear transfer embryos were cultured in co-culture system using B2, CR1aa, and CR2 at 20% O_2 and blastocysts were obtained (Cibelli et al., 1998 ; Kato et al., 1998 ; Vignon et al., 1998). In this study,

Table 2. Effect of O_2 concentration and co-culture on the development of embryos produced by cumulus cell nuclear transfer in Hanwoo

Gas phase[1]	Co-culture[2]	No. of oocytes enucleated	No. (%) of oocytes fused	No. (%) of oocytes developed to	
				2 cell	Blastocyst
5%O_2	+	101	67 (66.3)	56 (83.6)^a	22 (32.9)^a
	-	94	66 (70.2)	52 (78.8)^a	21 (31.8)^a
	Subtotal	195	133 (68.2)	108 (81.2)	43 (32.3)^c
20%O_2	+	93	47 (50.5)	29 (61.7)^b	7 (14.9)^b
	-	97	58 (60.0)	46 (79.3)^a	4 (6.9)^b
	Subtotal	190	105 (55.3)	75 (71.4)	11 (10.5)^d

^{a,b} Means with different superscripts within the same columns were significantly different ($p<0.05$).

^{c,d} Only subtotal means with different superscripts within the same columns were significantly different ($p<0.05$).

[1] 5% O_2 : 5% CO_2 , 5% O_2 , 90% N_2 and 20% O_2 : 5% CO_2 in air. Four replicates.

[2] Co-culture cell was the feeder layer of 1-1.2 x 10^5/ml cumulus cells pretreated with 10 ug/ml mitomycin C for 2.5 h.

Table 3. Reproductive performance of Hanwoo cows with transferred blastocysts produced by cumulus cell nuclear transfer

No. of cows transferred	No. of embryos transferred	No. (%) of cows pregnant at 100 days	Aborted[1]	Calved[2]
25	50	5 (20.0)	3	2

[1] Fetuses of three cows were aborted at 152, 229 and 258 days of gestation, respectively.

[2] One cow was delivered of twin and the other cow was delivered of a calf, but the latter calf died immediately after birth.

however, better development to blastocyst was obtained in the modified CR1aa at 5% O_2. This result was similar to the previous results that if fertilized embryos were cultured under lower O_2 atmosphere, better development could be obtained with or without co-culture according to culture media (Nakao and Nakasuji, 1990 ; Thompson et al., 1990 ; Voelkel and Hu, 1992 ; De Azambuja et al., 1993). Furthermore, the present result confirmed the result of Tervit et al. (1972) that an oxygen concentration around 5-10% was optimal for development of sheep and cattle embryos *in vitro*. Similar studies utilizing early mouse embryos demonstrated that 5% O_2 was optimal for development (Whitten, 1971 ; Quin and Harlow, 1978). However, other studies have not been able to demonstrate an effect of reduced oxygen concentration (5%) on the development of sheep embryos (Wright et al., 1976 ; Betterbed and Wright, 1985). Oxygen tension in the oviduct was about 5.3-7.9% in the rabbit and monkey (Mastroianni and Jones, 1965 ; Mass et al., 1976). These facts indicated that a low O_2 concentration could increase preimplantation bovine embryo development (Nagao et al., 1994 ; Lonergan et al., 1999). Free oxygen radicals were the most important factor to reduce development when embryos were cultured under 21% O_2 atmosphere. Free oxygen radicals are potent oxidants. Therefore, the accumulation or damage by these should be protected by antioxidants such as superoxide dimutase and thioredoxin (Noda et al., 1991 ; Nonogaki et al., 1991 ; Umaoka et al., 1992).

This study showed that there was no significant difference between with- and without- cumulus cell co-culture when the nuclear transfer embryos were cultured at 5% O_2 or 20% O_2, respectively. However, the development to blastocyst was higher when nuclear transfer embryos were cultured with cumulus cell co-culture at 5% O_2 than 20% O_2. This result was consistent with the study of Voelkel and Hu (1992) who reported that a higher development was obtained when the *in vitro* matured and fertilized bovine embryos were co-cultured with oviduct cells at 5% O_2 than 20% O_2, but, in contrast, a higher development was obtained when the embryos were co-cultured with buffalo rat liver cells at 20% O_2 than 5% O_2. However, this result was not similar to that of Nakao and Nakasuji (1990) who reported that if embryos were co-cultured with somatic cell, a higher development could be maintained even when the medium and gas phase were changed. Fukui et al. (1991) reported that without co-culture, higher atmospheric O_2 was toxic to embryos, since 5% O_2 supported much higher blastocyst development, but when the co-culture was used, 5% O_2 was insufficient and 20% O_2 was optimal. They suggested that one function of co-culture cells was to reduce O_2 tension to less toxic level, and they also illustrated the difficulty to establish optimal culture conditions in the presence of co-culture cells. These differences among experiments are not exactly understood, but it may be due to different media, co-culture cells, O_2 concentrations and culture drop used.

Using Hanwoo as a model, we compared the developmental potential of embryos reconstructed with nuclei from cycling and quiescent cells from cumulus cells (from cow) and the G0 state was evidenced in flow cytometer analysis. We did not observe any difference in the rate of blastocyst formation. We concluded that serum starvation was not a prerequisite for successful nuclear transfer from cumulus donor cell in Hanwoo.

The percentage of nucleus transfer bovine embryos developing to term was approximately 20% after transfer to recipient cows (Bondioli et al., 1990 ; Westhunsin et al., 1991 ; Kono et al., 1994). In this study, 5 of 25 (20%) recipient cows were pregnant at 100 days of gestation. The low pregnancy rate coupled with high abortion rate were obstacles to large-scale cloning in cattle (Kono et al., 1994). The birth weight of cloned calves was heavier than that of calves produced by *in vivo* method (Garry et al., 1996). This same large calf syndrome was reported for calves resulting from IVM-IVF-IVC (Kruip et al., 1997) and embryonic nuclear transfer cloning (Garry et al., 1996 ; Shiga et al., 1999). We need further researches to elucidate the causes of the oversized fetus and the low pregnancy rate during development of the cloned embryo/fetus.

In conclusion, this study showed that the optimal culture atmosphere was 5% CO_2, 5% O_2, 90% N_2 at 38.5°C. For better development to blastocyst, FBS should be added in the culture medium at post cleavage stage. The embryos reconstructed by enucleation of metaphase II oocytes and introduction of the cycling and quiescent cumulus donor cells in Hanwoo had develomental potential to term after embryo transfer to recipient cows.

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