Effect of Culture Conditions and Freezing Methods on Developmental Competence of Hanwoo Embryos Cultured *In Vitro*

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ABSTRACT

This study was carried out to examine on developmental competence of Hanwoo embryos cultured *in vitro* according to culture conditions and freezing methods. The *in vitro* developmental competence to blastocyst stage at Day 8 of culture in SOF was significantly ($p<0.05$) higher than that in CR1aa (30.3% vs. 18.4%). The *in vitro* developmental rate of morula and blastocysts cultured in group culture was significantly ($p<0.05$) higher than that in individual culture (41.4% and 36.0% vs. 21.1% and 10.5%, respectively). The cell number of Day 8 blastocysts in group culture was significantly ($p<0.05$) higher than that in the individual culture (120.1 ± 12.8 vs. 94.1 ± 12.1, respectively). The survival rates of frozen-thawed balstocysts that were exposed in 1.5 M ethylene glycol or 1.5 M ethylene glycol containing 0.1 M sucrose were 77.5% and 78.7%, respectively. The survival rates of blastocysts cultured for 48 h in slow freezing and vitrification was not significantly different (73.3 and 74.0%).

In conclusion, *in vitro* developmental competence of bovine embryos was influenced on the culture medium (SOF) and culture method (Group culture). Survival rate of frozen-thawed of bovine embryos was not influenced on freezing solutions and freezing methods.

(Key words : *in vitro* culture, bovine embryo, slow-freezing, vitrification)

INTRODUCTION

According to commercialization of bovine embryo transfer becomes possible, a lot of bovine embryos were needed, but the low developmental and survival rate of embryos by inappropriate culture system and freezing methods act on the obstacle factors of activation of bovine embryo transfer. although *in vitro* culture system and freezing methods of bovine embryos that can the mass-production of bovine embryos are established, the commercial bovine embryo transfer may be available regardless of estrous cycle of recipient cows.

Several investigators indicated that culture medium and culture methods are played on the developmental competences of bovine embryos cultured *in vitro*. TCM199 was used mainly as bovine embryo culture medium, but CR1aa and SOF has been using widely in recently (Nedamblea *et al.*, 2006; Holm *et al.*, 1999). Citrate stimulates fatty acids synthesis and an important role for compaction and blastocoel formation (Gary *et al.*, 1992). Sung *et al.* (2004) demonstrated a beneficial effect of sodium citrate and BSA on the development of bovine IVP embryo when embryos were cultured with somatic cells such as cumulus cells. Increasing the embryos density stimulates the rate of compaction (Stoddart *et al.*, 1996) and cavitation (Wiley *et al.*, 1986).

Many of calves has been produced by transfer of bovine frozen embryos, but bovine embryos cultured *in vitro* were more sensitive during freezing and thawing procedure than those of *in vivo* (Hasler *et al.*, 1995). Slow freezing and vitrification are commonly used to cryopreserve bovine embryo. For bovine embryo direct transfer, slow freezing is widely used and with the low concentration of cryoprotectants (Volkel and Hu, 1992). Vitrificaiton is the solidification of solution without ice crystal formation, and induced the glass state by high concentration of cryoprotectants (Kuwayama *et al.*, 1994). In recently, survival rates of bovine embryos after cryopreservation have been improved by vitrification. Several investigators have been reported that vitrifying of bovine embryos in small amount of vitrification solution with open pulled straw (OPS; Vajta *et al.*, 1999), Cryo-loop (Yeoman *et al.*, 2001; Lane *et al.*, 1999), glass micropipette (GMP; Cho *et al.*, 2002), and Gel loading

† 본 연구는 농촌진흥청 지역특화기술개발사업의 지원에 의해 이루어짐.
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tip (GLT; Tominaga and Hamada, 2001) were obtained higher survival rate than in controlled freezing. The gel loading tip seems to be the best in controlling the vitrification solution volume and handling numerous embryos because of the help of the micropipette (Tominaga and Hamada, 2001). The first introduction of mouse embryos was by slow freezing (Wittingham, 1971), and later applied to bovine embryos cultured *in vitro* (Wilmut and Rowson, 1973). The usefulness of low toxicity and fast permeation of ethylene glycol was demonstrated, and is suitable for direct transfer of bovine embryo (Voekel and Hu, 1992; Leibo, 1984).

In this study, we have examined on the developmental competence of Hanwoo embryos cultured *in vitro* according to culture conditions, freezing methods for establishment of mass-production of bovine embryos cultured *in vitro*.

MATERIALS AND METHODS

1. Oocyte Collection and *In Vitro* Maturation

Bovine ovaries from slaughtered cows were transported to the laboratory in saline {0.9% (w/v) NaCl} containing 100 μg/ml streptomycin and 100 μg/ml penicillin G at 28~30℃, and then washed three times in saline. Oocytes-cumulus cells complexed were aspirated from follicles 2 to 7 mm in diameter with a 10 ml disposable syringe attached to 18 G needle and placed into 50 ml conical tubes for 10 min. The COCs with a homogeneous cytoplasm and intact tight cumulus cell layers selected for *in vitro* maturation, and washed twice in aspiration medium and transferred into in groups of 50 per well dishes containing 500 μl of IVM medium. The medium used for oocyte maturation was TCM199 supplemented with 10% FBS, 10 μg/ml FSH, and 10 μg/ml LH. The immatured COC's were incubated for 22~24 h under paraffin oil at 39℃, 5% CO_2 in humidified air.

2. Sperm Preparation and *In Vitro* Fertilization

Frozen-thawed semen was loaded on the top of discontinous Percoll gradient (90%/45%) in a 15 ml conical tube and centrifuged for 20 min at 500 ×g at room temperature. The supernatants was discarded and centrifuged once in 2 ml SP-TALP at 300 ×g for 5 min. The pellet was resuspended 100 μl Fert-TALP supplemented with 5 μg/ml heparin, 10 μM penicilliamine, 15 μM hypotaurine and 1 μM epinephrine. Matured COCs were transferred into 100 μl IVF drops. The final concentrations of sperm were modulated at the $1\sim2\times10^6$ cells/ml. The gametes were cultured under paraffin oil for 18~20 h at 39℃, 5% CO_2 in humidified air.

3. *In Vitro* Culture

The presumptive zygotes were vortexed for 1 min in 0.5 ml IVC medium in a 15 ml conical tube. The zygotes were washed in IVC medium and cultured in CR1aa and SOF. One or 25 bovine embryo were cultured in each of 25 μl SOF under paraffin oil at 39℃ in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 in air for 8 days after fertilization. Blastocysts obtained were stained with Hochest 33342 to examine the total cell number of bovine blastocyts (Day 8).

4. Cryopreservation of Day 8 Blastocysts

The basic medium was consisted of D-PBS containing 10% FBS, and freezing media are consist of 1.5 M ethylene glycol and 1.5 M ethylene glycol containing 0.1 M sucrose in basic medium. Bovine blastocysts were washed three times in basic medium, equilibrated in each freezing medium for 10 min. The straw which loaded bovine embryos placed at −7℃ into the cryochamber of freezer (CL863, Australia). After 5 min, seeding was induced with supercooled forceps. Subsequently, the samples were cooled 0.5℃/min to −32℃ and plunged into liquid nitrogen. After several weeks, the straws were thawed in 30℃ water bath for 1 min. The straw were expelled into culture dish and diluted by washing the embryos three times in D-PBS containing 10% FBS. Following thawing and rehydration, the embryos were cultured in SOF containing 5% FBS for 48 h.

To vitrify Day 8 blastocysts, three to five embryos were washed in D-PBS containing 20% FBS (basic medium) and exposed to 50 μl equilibration solution consisted of 10% ethylene glycol (EG) and 10% DMSO in basic medium for 2 min at 37℃. The embryos were exposed to vitrification solution consisted of 20% EG, 20% DMSO and 0.6 M sucrose in basic medium for 30 sec at 37℃. The embryos loaded into GLT and plunged into liquid nitrogen. After several week, the straw were thawed by immersing the GLT in 37℃ water bath and expelled into 0.25 M sucrose for 1 min. The blastocysts were transferred into 0.13 M sucrose and cultured into SOF containing 5% FBS.

5. Statistical Analysis

Statistical analysis was performed with one-way ANOVA and a value of $p<0.05$ was chosen as an indication of statistical significance.

RESULTS

1. Developmental Competence of Bovine Embryos Cultured *In Vitro* according to Culture Media

In this experiment, the *in vitro* developmental competences of bovine embryos cultured in CR1aa and SOF was examined (Table 1). The cleavage rate of bovine zygotes that were cultured in the CR1aa and SOF were 82.4% and 84.2%, respectively. *In vitro* developmental competences of morula and blastocysts in the CR1aa and SOF and were 35.1%, 39.1% and 18.4%, 30.3%, respectively. The developmental competences of blastocysts cultured in the SOF were significantly ($p<0.05$) higher than that in the CR1aa.

2. Developmental Competence and Cell Number of Bovine Embryos by Individual and Group Culture

In this experiment, the *in vitro* developmental competence of bovine embryos cultured by individual and group culture was examined (Table 2). Developmental rate of morula and blastocysts cultured in the group culture was significantly ($p<0.05$) higher than that in the individual culture (41.4% and 36.0% vs. 21.1% and 10.5%, respectively). The velocity of development into blastocyst of the group culture was slightly faster than that of the individual culture.

The cell number of Day 8 blastocysts cultured in individual and group culture was examined (Table 3). The cell number of Day 8 blastocysts in the group culture was significantly ($p<0.05$) higher than that in the individual culture (120.1 ± 12.8 vs. 94.1 ± 12.1, respectively). Increasing embryo density in constant media volume was improved the developmental competence and cell number of bovine embryos.

3. Survival Rate of Frozen-thawed Bovine Embryos by Different Freezing Solution and Freezing Methods

In this experiment, the survival rate of Day 8 blastocysts was examined following exposure to different freezing solution (Table 4). This results shown that survival rate of post-thawed bovine embryos exposure to 1.5 M EG or 1.5 M EG containing 0.1 M sucrose freezing solution was 77.5% and 78.7%, respectively. The degeneration of bovine embryos in 1.5 M EG or 1.5 M EG containing 0.1 M sucrose freezing solution was 22.5% and 21.2%, respectively. There were no significantly ($p<0.05$) differences between 1.5 M EG or 1.5 M EG containing 0.1 M sucrose freezing solution. The survival rate of bovine blastocysts following slow freezing and vitrification was exa-

Table 1. Developmental ability of bovine embryos cultured *in vitro* by different culture media

Culture media	No. of oocytes used	No. of cleaved oocytes	No. and (%) of embryos developed to	
			Morula	Blastocyst
CR1aa	245	202 (82.4)	86 (35.1)	45 (18.4)[b]
SOF	260	219 (84.2)	103 (39.1)	79 (30.3)[a]

[a,b] Values with different superscripts in the same column are significantly different ($p<0.05$).

Table 2. Effects of individual and group culture on development of bovine embryos cultured *in vitro*

Culture methods	No. of embryos cultured	No. and (%) of embryos developed to	
		Morula	Blastocyst
Individual	190	40 (21.1)[a]	20 (10.5)[a]
Group	220	91 (41.4)[b]	79 (36.0)[b]

[a,b] Values with different superscripts in the same column are significantly different ($p<0.05$).

Table 3. Cell number of bovine embryos cultured *in vitro* according to culture methods

Culture methods	No. of embryos used	Cell number of blastocyst at Day 8	
		Mean±SD	Range
Individual	15	94.1±12.1[a]	80~110
Group	14	120.1±12.8[b]	90~140

[a,b] Values with different superscripts in the same column are significantly different ($p<0.05$).

Table 4. Survival rates of cryopreserved bovine embryos cultured *in vitro* by different freezing solution

Kinds of freezing solutions	No. of embryos used	No. and (%) of embryos	
		Survivability	Degeneration
1.5 M EG	40	31 (77.5)	9 (22.5)
1.5 M EG + 0.1 M Suc	47	37 (78.7)	10 (21.2)

mined (Table 5). The survival rate of bovine blastocysts following slow freezing in 1.5 M EG and vitrification (GLT) was 73.3% and 74.0%, respectively. There were no significantly differences between slow freezing and vitrification.

DISCUSSION

In this study, *in vitro* developmental competence to blastocyst stage in SOF was significantly ($p<0.05$) higher than that in CR1aa. Holm *et al.* (1999) reported that balstocyst formed earlier in SOF with inclusion of serum compared to TCM199 co-culture with granulose cells. SOF with sodium citrate stimulates fatty acids synthesis and compaction and blatocoel formation (Gary *et al.*, 1992). Addition of myo-inositol to SOF with citrate and PVA can be improved blastocyst development (Holm *et al.*, 1999), and behaves as an osmolyte together with glycine (Li and Foote, 1995). Nedambalea *et al.* (2006) reported that developmental rate of bovine embryos cultured in SOF was higher than those in CR1aa and similar to our results.

The development of embryo cultured *in vitro* is dependent on embryo density and so embryo culturing in small volume was faster developmental competence than that in large volume (O'Neill, 1997; Lane and Gardner, 1992; Paria and Dey, 1990). Increasing the embryos density stimulates the rate of compaction (Stoddart *et al.*, 1996), cavitation (Paria and Dey, 1990; Wiley *et al.*, 1986) and increasing of the cell number (Stoddart *et al.*, 1996). Optimal blastocyst formation rate occurred when embryos were cultured 165 μm distance between individual bovine embryos (Gopichdran and Leese, 2006). The distance between individual bovine embryos in culture influences their development, in terms of blastocysts formation, cell number and metabolism (Gopichandran and Leese, 2006). Lane and Gardner (1992) show that increasing culture medium volume reduced the cell number of blastocysts. This result is similar to the results of several investigators (O'Neill, 1997; Lane and Gardner, 1992; Paria and Dey, 1990).

Addition of high concentration of sucrose as an osmotic buffer to freezing solution is detrimental to frozen embryos, which may develop membrane permeability problems (Smogsasen *et al.*, 1995; Szell and Shelton, 1986; Leibo, 1984). The cooling rate of GLT (200 μm inner diameter) during vitrification is expected to be over 25,000℃/min, because the cooling rate of OPS (700 μm inner diameter) has been reported to be 22,500℃/min (Tominaga and Hamada, 2001). Moreira and Metelo (2005) provide evidence that the ZP properties of *in vitro* produced embryos are directly related to the viability of bovine embryos after slow freezing and vitrification. The step of cryopreservation cause the alteration of ZP, and this cause the irreversible damage on further developmental competence of bovine embryos. Vitrification results in a higher survival rate than that of slow freezing, regardless of the presence or absence of serum culture medium (Mucci *et al.*, 2006). Owing to ice crystal formation, slow freezing of bovine embryos produced *in vitro* lowered the survival rates compared with their *in vivo* counterparts (Kasai *et al.*, 2002).

Table 5. Survival rates of bovine embryos cultured *in vitro* by slow freezing or vitrification

Freezing methods	No. of embryos used	No. and (%) of embryos	
		Survivability	Degeneration
Slow freezing	60	44 (73.3)	16 (26.7)
Vitrification	50	37 (74.0)	13 (26.0)

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(접수일: 2007. 12. 1 / 채택일: 2007. 12. 24)