In vitro Development of Somatic Cell Nuclear Transferred Bovine Embryos Following Activation Timing in Enucleated and Cryopreserved MII Oocytes

Park, S. P.[†], E. Y. Kim, S. G. Kim, Y. J. Lee, K. S. Kil, S. Y. Park, J. Y. Yoon, C. H. Lee, K. S. Chung[1] and J. H. Lim[2]

Maria Infertility Medical Institute/Maria Biotech

탈핵 후 동결한 MII 난자의 활성화 시기가 체세포 핵치환 이후 소 난자의 체외발달에 미치는 영향

박세필[†] · 김은영 · 김선균 · 이영재 · 길광수 · 박세영 · 윤지연 · 이창현 · 정길생[1] · 임진호[2]

마리아 기초의학연구소/마리아 생명공학연구소

ABSTRACT

This study was to evaluate the *in vitro* survival of bovine enucleated MII (eMII) oocytes according to minimun volume cooling (MVC) freezing method and activation timing, and their *in vitro* development after somatic cell nuclear transfer (SONT). *In vitro* matured bovine oocytes for 20 h were stained with 5 μg/ml Hoechst, and their 1st polar body and MII plate were removed by enucleation micropipette under UV filter. Also, eMII oocytes were subjected to activation after (group II) and before (group III) vitrification in 5 μM ionomycin added CR1aa medium for 5 min. For vitrification, eMII oocytes were pretreated with EG10 for 5 min, exposed to EG30 for 30 sec and then directly plunged into LN$_2$. Thawing was taken by 4-step procedures at 37℃. Survived eMII oocytes were subjected to SONT with cultured adult bovine ear cells. Reconstructed oocytes were cultured in 10 μg/ml of cycloheximide and 2.5 μg/ml of cytochalasin D added CR1aa medium for 1 h, and then in 10 μg/ml of cycloheximide added CR1aa medium for 4 h. Subsequently, the reconstructed oocytes were incubated for 2 days and cleaved embryos were further cultured on cumulus-cell monolayer drop in CR1aa medium for 6 days. Survival rates of bovine vitrified-thawed eMII oocytes in group II (activation after vitrification and thawing) and III (activation before vitrification) were 81.0% and 84.9%, respectively. Fusion rates of cytoplasts and oocytes in group II and III were 69.0% and 70.0%, respectively, and their results were not different with non-frozen NT group (control, 75.2%). Although their cleaved rates (53.4% and 58.4%) were not different, cytoplasmic fragment rate in group II (32.8%) was significantly higher than that in group III (15.6%)(P<0.05). Also, subsequent development rate into >morula in group II (8.6%) was low than that in group III (15.6%). However, *in vitro* development rate in group III was not

[†] Corresponding author : Maria Infertility Medical Institute/Maria Biotech, Seoul 130-110, Korea. Tel: (02) 924-8757 Fax: (02) 924-9083, E-mail: sppark@mariababy.com
[1] 건국대학교 축산대학 (College of Animal Husbandry, Konkuk University)
[2] 마리아 병원 (Maria Hospital, Seoul 130-110, Korea)

different with that in control (24.8%). This result suggested that MVC method was appropriate freezing method for the bovine eMII oocytes and vitrified eMII oocytes after pre-activation could support *in vitro* embryonic development after SONT as equally well as fresh oocytes.

(Key words: Enucleated bovine oocytes, MVC freezing, Activation timing, Somatic cell nuclear transfer, *In vitro* development)

I. INTRODUCTION

Following the report regarding the cloned animals using sheep (Wilmut et al., 1997), so called "Dolly", similar strategies for producing cloned animals in cattle (Kato et al., 1998; Wells et al., 1999), goats (Baguisi et al., 1999), mice (Wakayama et al., 1998, 1999), and pigs (Polejaeva et al., 2000; Onishi et al., 2000) were successfully tried. However, cloning efficiency is considerably variable, which may be related to the different nuclear transfer protocols employed in each laboratory. The methodology used for cloned embryos in each of these species is essentially similar: somatic cells, diploid donor nuclei, have been transplanted into enucleated MII oocytes that are activated on or after transfer. To date, Colman (2000) report that the efficiency of somatic cell nuclear transfer (SONT), when measured as development to term as a proportion of oocytes used, has been very low (1~2%).

In SONT, if the cryopreserved bovine oocytes were to be used easily as recipient cytoplasts, it would alleviate the logistical problems associated with matching the availability of donor cells. It was reported that frequent loss of post-thaw surviving oocytes at enucleation and the lower fusion rate in SONT using cryopreserved bovine MII oocytes (Ito et al., 1999). Also, another problems were demonstrated that associated with chilling and freezing injury, mechanical damage of frozen oocytes during SONT (Aman and Parks., 1994; Ito et al., 1998), and the lack of the effect of cytochalasin B during cryopreservation (Dorbrinsky et al., 1997; 1998).

Liu et al. (1998) and Ito et al. (1999) reported that parthenogenetic activation-induced changes in enucleated oocytes, such as membrane transport system, protein patterns, and microtubular organization, are involved in the tolerance of oocytes to cryoinjuries. In the reason of poor cryosurvival of oocytes, most of current SONT study in cattle must rely on freshly collected oocytes. According to Martino et al. (1996), vitrification is substantially better for cells that have high chilling sensitivities such as bovine oocytes and embryos, higher concentration of cryoprotective agents and ultra rapid cooling speed are aid to *in vitro* survival of frozen-thawed bovine oocytes. Recently, vitrification methods combined with various ovum containers (EM-grid, narrow and small wide straw, droplet without container and minimum volume cooling straw) and minimum volume (1~2 μl) of freezing solution, which has high cooling capacity, were continually introduced for simple and efficient cryopreservation of bovine *in vitro* matured oocytes (Martino et al., 1996; Vajta et al., 1998; Papis et al., 1999; Dinnyes et al., 2000; Kim et al., 2001). We also reported that *in vitro* matured bovine oocytes were successfully cryopreserved by a vitrification using minimum volume cooling (MVC) method and those oocytes were developed into full term after *in vitro* fertilization and embryo transfer (Kim et al., 2001).

The objective of our study was to evaluate the *in vitro* survival of bovine enucleated MII (eMII) oocytes according to MVC freezing method and activation timing, and their *in vitro* development after SONT.

II. MATERIALS AND METHODS

1. Preparation of Recipient Oocytes

Bovine ovaries were obtained from a local slaughterhouse, transported in saline at $32 \sim 37\,^{\circ}\mathrm{C}$ to the laboratory within $2 \sim 4$ h after slaughter and washed in $39\,^{\circ}\mathrm{C}$ TL-HEPES (Parrish et al., 1988). Cumulus oocyte complexes (COCs) were collected from visible follicles ($2 \sim 6$ mm) of ovaries and cultured in maturation medium which consisted of TCM-199 (Gibco)+10% (v/v) FBS (Gibco) supplemented with sodium pyruvate (0.2 mM), follicle-stimulating hormone (1 μg/ml), estradiol-17 β(1 μg /ml) at $39\,^{\circ}\mathrm{C}$, 5% CO_2 incubator.

For enucleation, oocytes were exposed to 5 μg/ml Hoechst 33342 (Sigma) for 10 min. The metaphase chromosome was removed in TCM-HEPES containing 5 μg/ml cytochalasin B (Sigma) and visualized under epifluorescence microscope (Olympus, Japan) to confirm the absence of chromatin.

2. Experimental Design

Bovine oocytes with a visible first polar body at 20 h after the start of maturation were enucleated, fused with donor cells (bovine ear cells) by two DC eletropulses, and parthenogenetically activated for 5 h. The reconstituted oocytes were cultured for 8 days to allow blastocyst development (group I: non -frozen control). Vitrification procedure, MVC method, was carried out immediately after enucleation (group II) or after enucleation and activation (group III). For group II and III, activation of enucleated oocytes was treated with 5 μM ionomycin for 5 min. The vitrified-thawed oocytes were activated for an additional 5 h after nuclear transfer.

3. Preparation of Freezing Solution

The basic medium for pretreatment, vitrification, and dilution was 10% FBS added Dulbecco's phosphate-buffered saline (D-PBS, Gibco). The pretreatment solution contained 10% ethylene glycol (EG10). The vitrification solution (VS) consisted of 30% ethylene glycol and 0.5 M sucrose (EG30). For serial dilution after thawing, 1.0, 0.5, 0.25 or 0.125 M sucrose and 10% FBS added D-PBS were prepared.

4. Vitrification and Thawing

Oocytes were frozen-thawed according to the procedures reported previously (Kim et al., 2001). Oocytes were washed three times in TALP-HEPES and then they were suspended in D-PBS before the each treatment.

Freezing procedures were performed at room temperature of $25 \sim 26\,^{\circ}\mathrm{C}$. For the vitrification, oocytes were pretreated in EG10 for $5 \sim 10$ min, exposed in EG30 for 30 sec, and then each oocytes were individually put on the inner wall of 0.25 ml mini straw (IMV, L'Aigle, France) with a minimum volume ($<2\,\mu$l) of VS (namely, designated as MVC method). Straws were directly plunged into LN_2. A mean number of oocytes put on one MVC straw was ten.

In thawing, cryoprotectants were removed by 4-step procedures at $37\,^{\circ}\mathrm{C}$. MVC straws storaged in LN_2 were moved rapidly into 1.0 M sucrose. Then recovered oocytes were transferred to 0.50 M sucrose, 0.25 M sucrose, and 0.125 M sucrose. Each step needs for 1 min. Then, the number of surviving oocytes having morphologically normal plasma membrane was recorded.

5. Nuclear Transfer

Female adult fibroblasts were obtained from bovine ear tissue of Hanwoo (Korean native cattle). The isolated tissues were washed three times in PBS, cut into small pieces and dispersed by exposure into 0.1% collagenase for 1 h. The cell suspension was then transferred into 75 mm culture flask

containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. The cells were cultured until sub-confluence at 39℃ in 5% CO_2 in air, and then frozen using 10% dimethyl sulfoxide added culture medium and stored in liquid nitrogen. For experiments, the cells were thawed and cultured for up to three passages.

Single donor cells (bovine ear cells) were transferred into the perivitelline space of fresh or vitrified -thawed eMII oocytes. Manipulated eggs were placed in a 0.5 mm round wire, stainless steel electrode chamber filled with 0.3 M mannitol containing 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 0.05 mg/ml of BSA and treated by two direct current (DC) pulses of 1.5 kV/cm for 30 μsec in order to initiate their fusion. They were then cultured in CR1aa medium supplemented with 10 μg/ml cycloheximide (C-7698, Sigma) and 2.5 μg/ml cytochalasin-D (C-8273, Sigma) for 1 h and in CR1aa medium supplemented with only 10 μg/ml cycloheximide for 4 h. The reconstituted NT oocytes were cultured in CR1aa medium (Rosenkrans et al., 1993). After 48 h of culture, only cleaved embryos were further co-cultured on cumulus cell monolayer drop in CR1aa supplemented with 10% FBS for another 6 days.

6. Evaluation of Oocyte Survival after Thawing

In vitro survival in each treatment was assessed at 2 h after thawing showed no differences of

cytoplasmic appearance and membrane integrity; no sign of lysis, membrane damage, swelling or vacuolization (Fig. 1).

7. Statistical Analysis

The data from at least three replications were pooled. Differences in the percentages of developed to particular stages were estimated by Student's *t*-test.

Ⅲ. RESULTS

In vitro survival rates of bovine vitrified-thawed eMII oocytes in group II and III were 81.0% and 84.9%, respectively. As shown in Fig. 1, vitrified -thawed eMII oocytes in group II (Fig. 1A') and III
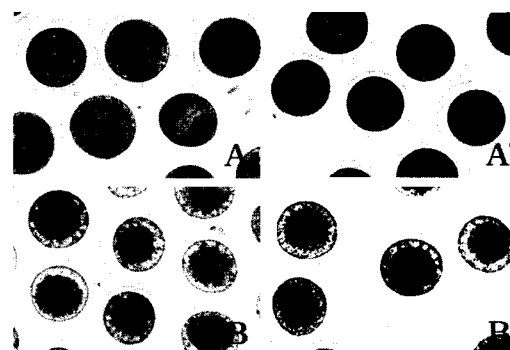

Fig. 1. Morphology of bovine eMII oocytes before (A, B) and after (A', B') freezing using MVC method. A and A': group I. B and B': group III. ×200.

Table 1. *In vitro* development of vitrified-thawed bovine eMII oocytes after somatic cell nuclear transfer

Group*	No.	Survived (%)	Fused/NT (%)	Cleaved (%)	>Morula (%)
I	156	–	109/145(75.2)	69(63.3)	27(24.8)[a]
II	147	119(81.0)	58/ 84(69.0)	31(53.4)	5(8.6)[b]
III	172	146(84.9)	77/110(70.0)	45(58.4)	12(15.6)[ab]

Non-frozen control group, II: Activated eMII group after vitrification and thawing,
III: Activated eMII group before vitrification.
[a,b] Different superscripts denote significant differences (P<0.05)

Table 2. Cytoplasmic fragment in vitrified-thawed bovine eMII oocytes according to activation timing

Group*	No.	Survived (%)	Fused/NT (%)	Cleaved (%)	Cytoplasmic fragment(%)
II	147	119(81.0)	58/ 84(69.0)	31(53.4)	19(32.8)^a
III	172	146(84.9)	77/110(70.0)	45(58.4)	12(15.6)^b

* II: Activated eMII group after vitrification and thawing, III: Activated eMII group before vitrification.
^{a,b} Different superscripts denote significant differences ($P<0.05$).
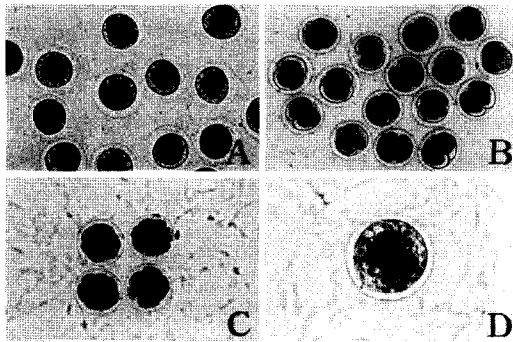

Fig. 2. *In vitro* development of vitrified-thawed bovine eMII oocytes derived from group III after nuclear transfer. A. Oocytes after thawing (×150). B. Cleaved embryos at day 2 after nuclear transfer (×150). C. *In vitro* developed embryos at day 5 after nuclear transfer (×150). D. *In vitro* developed blastocyst at day 8 after nuclear transfer (×200).

(Fig. 1B') were not different in cytoplasmic appearance and membrane integrity; no sign of lysis, membrane damage, swelling or vacuolization, compared to eMII oocytes before freezing (Fig. 1A and 1B), repectively. After SONT of post-thaw surviving oocytes, fusion and *in vitro* developmenta rates (cleavage at day 2 and > morula at day 8) in group II (69.0, 53.4 and 8.6%, respectively) were not significant different compared to those in group III (70.0, 58.4 and 15.6%, respectively) (Table 1). However, fusion and *in vitro* development rates in frozen-thawed eMII groups were not different from those in non-frozen control NT group (75.2, 63.3

and 24.8%), except the significantly low >morula rates in group II (8.6%). Also, the lower developmental potential obtained in group II (8.6%) than that in group III (15.6%) might be related to the significantly higher rate of cytoplasmic fragment in group II (32.8%) (Table 2). As shown in Fig. 2, vitrified eMII oocytes after pre-activation of group III could support *in vitro* embryonic development after SONT.

IV. DISCUSSION

The major finding of this study is that vitrified -thawed eMII oocytes can be used successfully as recipients for SONT as equally well as fresh oocytes. This finding has important implications for nuclear transfer research and establishment of ovum banking for NT. Previously, it has been reported that after *in vitro* fertilization (IVF), frozen-thawed mature bovine oocytes could be developed to the blastocyst stage (Lim et al., 1991). Since then, several teams have investigated the possibility of using cryopreserved bovine oocytes. However, oocytes survival and subsequent blastocyst development have remained low. Fortunately, recent development in vitrification methods have substantially increased the success rates in blastocysts derived from IVF or NT using frozen-thawed bovine MII oocytes (Kubota et al., 1998; Ito et al., 1999; Dinnyes et al., 2000; Kim et al., 2001).

In our study, the rates of *in vitro* survived oocytes

having morphologically normal plasma membrane after thawing in group II (frozen after enucleation) and group III (frozen after enucleation and activation) were 80.9% and 84.9%, respectively (Table 1). The relatively high rates of cryosurvival following vitrification of bovine oocytes can be attributed to several factors. Our MVC method achieved a high cooling rate by using MVC straw for eMII oocytes and improved heat exchange through by direct contact with MVC straw surface and LN_2, where the oocytes are loaded in an extremely low volume ($< 2 \mu l$) on to the wall of a 0.25 ml straw (Kim et al., 2001). In previous experiments, some investigators also reported that a minimum volume of vitrification solution into direct contacted with LN_2 was proven successful by eliminating the insulation effect of the container wall, and developmental capacity of frozen-thawed oocytes could be increased by reducing the time to traverse the damaging temperature (Papis et al., 1999; Yang and Leibo, 1999; Dinnyes et al., 2000; Kim et al., 2001).

In producing NT zygotes from frozen-thawed oocytes, Kubota et al. (1998) reported that enucleation of matured oocytes prior to freezing resulted in no development advantages compared to that of matured oocytes. Whereas Ito et al. (1999) reported that NT zygotes derived from frozen-thawed oocytes after enucleation and activation exhibited a higher development rates than those from other groups. In the present study, once oocytes survived beyond somatic cell (bovine ear cell) injection, fusion and cleavage rates in group II (69.0 and 53.4%) were no siginficant difference compared to those in group III. However, developmental potential into blastocysts of group III zygotes was not different to that of group I (non-frozen control), except for group II zygotes. Vitrified eMII oocytes without activation (group II) presented significantly higher rates of cytoplasmic fragment than vitrified eMII oocytes after activation (group III). Thus, we

presumed that the low *in vitro* development rate of eMII oocytes in group II was caused to their high cytoplamic fragment after NT. These results suggested that parthenogenetic activation-induced changes in enucleated oocytes, such as membrane transport system, protein patterns, and microtubular organization, are involved in the tolerance of oocytes to cryoinjuries (Liu et al., 1998; Ito et al., 1999).

From the results obtained in the present study, we concluded that MVC method was appropriate freezing method for the bovine enucleated MII oocytes, vitrified eMII oocytes after pre-activation could support *in vitro* embryonic development after SONT as equally well as fresh oocytes.

V. 요 약

본 연구는 체세포 핵치환에 탈핵 후 동결한 소 미수정란을 사용함에 있어서, MVC 초자화 동결방법과 탈핵난자의 활성화시기가 융해 후 생존율과 핵치환 이후 체외 발달에 미치는 영향을 조사하고자 실시하였다. 체외에서 20시간 동안 체외성숙된 소 미수정란은 수핵란으로 사용하기 위하여 5 μg/ml hoechst 처리 후, 형광현미경하에서 핵을 제거하였다. 본 실험은 세 그룹으로 나누어 실행되었다. Group I은 동결하지 않고 핵치환을 한 대조군이며, group III와 group II는 핵이 제거된 난자를 MVC 방법으로 동결하기 전과 후에 활성화 처리 (5 μM의 ionomycin에 의해서 5분간 처리) 한 군이다. 초자화 동결을 위해서는 group II와 group III의 탈핵란은 EG10에서 5~10분간 전처리하고 EG30에서 30초간 노출하여 액체 질소에 침지하였다. 융해는 37℃에서 4단계로 이루어졌다. 실험군은 모두 소 귀세포를 이용하여 핵치환을 실시하였으며, 전핵을 유도하기 위한 활성화를 위해서는 10 μg/ml cycloheximide와 2.5 μg/ml cytochalasin D가 첨가된 CR1aa 배양액에서 1시간, 이후 10 μg/ml cycloheximide가 들어있는 CR1aa 배양액에서 4시간 동안 배양하였다. 활성화 처리가 끝난 난자들은 CR1aa 배양액에서 2일간 배양하여 난할이 유도된

난자만을 선별하여 난구세포와 7일 동안 공배양하였다. 동결 융해 이후 group II와 group III의 탈핵된 소 미수정란의 체외 생존율은 81.0%와 84.9%로 유의적인 차이가 없었다. 체세포와 수핵란과의 융합율도 각각 69.0%와 70.0%로 대조군 (75.2%)과도 유의적인 차이를 나타내지 않았다. 난할율은 53.4%와 58.4%로 group II와 group III간에 유의적인 차이를 나타내지 않았지만 group II의 분할된 세포질을 가진 이상난자의 비율이 group III보다 유의하게 높게 나타났다 (P<0.05). 또한, morula 이상으로 발달율도 group II (8.6%) 에서 group III (15.6%)보다 낮은 결과를 얻었다. 하지만 group III (15.6%)의 체외 발달율은 대조군 (24.8%)과 유의한 차이를 없었다. 따라서, MVC 동결 방법은 탈핵된 소 미수정란을 동결하기에 적합한 방법이며, 탈핵후 activation을 유도하고 초자화 동결한 난자는 동결하지 않은 신선란과 동일하게 체세포 핵치환에 유용하게 이용될 수 있으리라 사료된다.

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