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Establishment of Bovine Ovum Bank : I. Full Term Development of Vitrified *In Vitro* Matured Hanwoo (Korean Cattle) Oocytes by Minimum Volume Cooling (MVC) Method

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소 난자 은행 설립 : Ⅰ. MVC 방법으로 초자화 동결된 한우 미성숙 난자의 개체 발생능 조사

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ABSTRACT

This study was to test whether *in vitro* matured Hanwoo oocytes can be successfully cryopreserved by a new vitrification procedure using MVC method. For the vitrification, oocytes were pretreated in 10% ethylene glycol (EG10) for 5~10 min, exposed in EG30 for 30 sec, each oocyte was individually put on the inner wall of 0.25 ml straw, and then straws were directly plunged into LN_2. Thawing was taken by 4-step procedures [1.0 M sucrose (MS), 0.5 MS, 0.25 MS, and 0.125 MS] at 37℃. *In vitro* developmental capacity (survival, cleavage (≥2-cell) and blastocyst rates) in vitrified group was no significant difference compared to that in other treatment groups (exposed; 100.0, 74.4, 32.3% and control; 100.0, 78.3, 36.3%); high mean percentage of oocytes (91.2%) was survived, 69.4% of them were cleaved and 27.9% of cleaved embryos were developed to blastocyst. Especially, after transfer of *in vitro* developed embryos in vitrified group, four of six recipient animals were pregnant and three of them were ongoing-pregnant by manual palpation at 250 days after transfer. This result demonstrates that MVC method is very appropriate freezing method for the Hanwoo *in vitro* matured oocytes and that ovum bank can be maintained efficiently by MVC cryopreservation method.

(Key words: Bovine *in vitro* matured oocyte, Vitrification, Thawing, *In vitro* survival, Full term development)

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I. INTRODUCTION

If oocytes could be successfully cryopreserved, an ovum bank could be established, and the techniques for advancing of genetic improvement could be promoted. Also, it may help researchers compensate for the obstacles they face through fluctuations in oocyte availability and seasonal variations in quality. Due to the sensitivity of low temperatures, cryopreservation of bovine oocytes remains a difficult task, despite the efforts of numerous research groups (Glass & Voelkel, 1990; Lim et al., 1991; Shellander et al., 1994). Only a limited number of publication reported blastocyst and subsequent calf development from cryopreserved oocytes (Fuku et al., 1992; Hamano et al., 1992; Otoi et al., 1992; Vajta et al., 1998). Since the first report of success for vitrification of mammalian embryos on 1985 by Rall & Fahy, many successes of vitrification have been reported in many species including human. In the viewpoint, vitrification is substantially better for cells that have high chilling sensitivities such as bovine oocytes and embryos (Martino et al., 1996). These higher concentration of cryoprotective agents and ultra rapid cooling speed are aid to *in vitro* survival of frozen-thawed bovine oocytes (Martino et al., 1996). Recently, vitrification methods combined with various ovum containers (EM- grid, narrow and small wide straw, and droplet without container) 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).

In this paper, we indicated the successful result on *in vitro* matured Hanwoo oocytes which is firstly cryopreserved by a new vitrification procedure using minimum volume cooling (MVC) method, which was demonstrated by Hamawaki (1999) for bovine blastocysts. It has merits that all treatment procedures are very simple and easy, and reliable. Also, we reported in this paper the full term development after transferred embryos followed by *in vitro* development of vitrified Hanwoo *in vitro* matured oocytes by MVC method.

II. MATERIALS AND METHODS

1. Collection of Oocytes and *In Vitro* Maturation (IVM)

Hanwoo (Korean cattle) ovaries were collected from a slaughterhouse and were transported within 2 to 4 h to the laboratory in 32~37℃. Cumulus oocyte complexes (COCs) were collected from visible follicles (2~6 mm) of ovaries, washed with TALP-HEPES (Parrish et al., 1988) and cultured into maturation medium composed of TCM-199 (Gibco) + 10% (v/v) FBS (Gibco) supplemented with sodium pyruvate (0.2 mM), follicle-stimulating hormone (1 μg/ml), estradiol-17β (1 μg/ml), and gentamycin (25 μg/ml) at 39℃, 5% CO_2 incubator. *In vitro* matured COCs were divided into three groups according to experimental purpose; control, exposure to cryoprotectant and freezing.

2. Preparation of Pretreatment, Vitrification and Dilution Solutions

The solutions for pretreatment, vitrification, and dilution were prepared using Dulbecco's phosphate-buffered saline (D-PBS, Gibco) plus 10% FBS. The pretreatment solution contained 10% ethylene glycol (EG10). The vitrification solution (VS) consisted of 30% ethylene glycol and 0.5 mol/L sucrose (EG30). The solutions for dilutions were made of 1.0, 0.5, 0.25 and 0.125 mol/L sucrose.

3. Vitrification and Thawing

After incubation for 22 h in IVM medium, oocytes were partially stripped of their cumulus cells by treatment with 0.1% hyaluronidase followed by pipetting. Oocytes were washed three times in TALP-HEPES and then they were suspended in D-PBS before the each treatment. MVC straws were prepared by partial cutting of straw about 1 cm length.

Freezing procedures were performed at room temperature of 25~26℃. For the vitrification, oocytes were pretreated in EG10 for 5~10 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). And 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℃. MVC straws storaged in LN_2 were moved rapidly into 1.0 M sucrose (S). And then recovered oocytes were transferred to 0.50 MS, 0.25 MS and 0.125 MS. Each step needs for 1 min Especially, in exposure group, all treatment except freezing procedure was done the same method as freezing group.

4. *In Vitro* Fertilization (IVF) and *In Vitro* Culture (IVC)

After being recovered from the exposure and

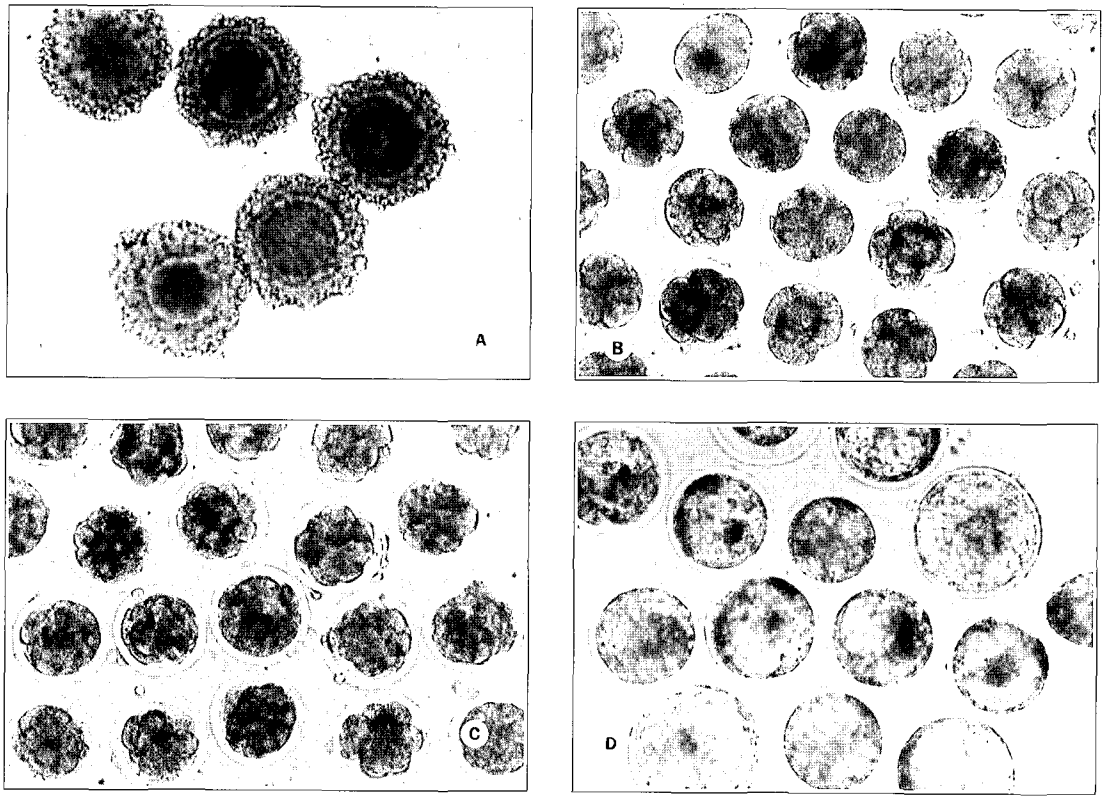

Fig. 1. *In vitro* survival and *in vitro* development of vitrified *in vitro* matured Hanwoo oocytes using MVC method. (A) Inseminated oocytes after thawing. (B) Cleaved embryos at day 3 after IVF. (C) *In vitro* developed embryos at day 5 after IVF. (D) *In vitro* developed blastocysts at day 8 after IVF. × 150.

thawing, the oocytes were incubated in fertilization drop for 30 min, and then they were subjected to IVF (Fig 1A). For IVF, highly motile sperm recovered from frozen-thawed and good quality Hanwoo semen (donated from Hanwoo Improvement Center, NLCF) separated on a discontinuous percoll column and heparin (2 μg/ml) and PHE (18.2 μM Penicillamine, 9.1 μM Hypotaurine and 1.8 μM Epinephrine) were added in fertilization drop. From day 2 after IVF, cleaved embryos were co-cultured in cumulus monolayer cell drop added CR1 (Charles Rosenkrans, 1993) medium supplemented with 10% FBS. Cumulus cell drop was prepared with the recovered cumulus cells from bovine *in vitro* matured oocytes before each treatment.

5. Evaluation of Oocyte Survival

In vitro survival in each treatment was assessed, at day 1 after IVF, with oocytes showed no difference of cytoplasmic appearance and membrane integrity; no sign of lysis, membrane damage, swelling, vacuolization, degeneration or leakage of the cellular content. Fertilization ($\geq$ 2-cell) and blastocyst formation were determined on day 2 and day 8 after IVF (Fig. 1B, 1D), respectively.

6. Embryo Transfer, Pregnancy Diagnosis and Calving

In vitro produced Hanwoo blastocysts after vitrification-thawing were transferred into recipients. Recipients were used for the nonsurgical embryo transfer after natural estrus. The corpus luteum was examined by palpation per rectum for morphological quality and no abnormal structures on the ovary or uterus immediately before transfer of the embryos. Pregnancies were confirmed at first when recipient cows did not return to the subsequent estrus cycle, and later by manual palpation per rectum on day 45, 90, 110, and 250.

7. Statistical Analysis

Difference in the rates of fertilization and developmental capacity among treatment group was compared using the Chi-square test.

Ⅲ. RESULTS

This study was to examine whether *in vitro* matured Hanwoo oocytes can be successfully cryopreserved by a new vitrification procedure using MVC method. When the *in vitro* survival of *in vitro* matured Hanwoo oocytes according to the exposure of cryoprotectants (in EG10 for 5~10 min and EG 30 for 30 sec at 25℃) was examined, significant toxicity was not showed in the exposure group (100.0, 74.4, 32.3%) compared to that of control group (100.0, 78.3, 36.3%). In addition, when those Hanwoo oocytes were vitrified using MVC method, there were no significant differences

Table 1. *In vitro* development of vitrified *in vitro* matured Hanwoo oocytes using MVC method (r=3)

Treatment	No. of examined oocytes	No.(%) of survived oocytes	No. (%) of	
			2-cell on day 2	Blastocysts on day 8
Control	130		102 (78.3)*	37 (36.3)*
Exposed	125	125 (100.0)	93 (74.4)*	30 (32.3)*
Freezing	204	186 (91.2)	129 (69.4)*	36 (27.9)*

* Not significantly different

Table 2. Pregnancy rates of vitrified *in vitro* matured Hanwoo oocytes using MVC method

Items	No.	Percent
No. of transferred cows	6	
No. of transferred embryos*	22 (3 or 4/each)	
No. of pregnancy cows (8 months after ET)	4/6	66.7%
No. of term	3/6	50.0%

* Day 7 embryos after IVF

in their developmental capacity compared to those of other treatment group; high mean percentage of oocytes (91.2%) was survived, 69.4% of them were cleaved and 27.9% of cleaved embryos were developed to blastocyst (Table 1). After transfer of day 7 embryos in vitrified group, four of six recipient animals did not return to the subsequent estrous cycle, and three of them were ongoing-pregnant by manual palpation at 250 days after transfer, respectively (Table 2).

Ⅳ. DISCUSSION

In this study, we first demonstrated that *in vitro* matured Hanwoo oocytes can be successfully cryopreserved by a new vitrification using MVC method and those oocytes can be developed into full term. It suggested that an ovum bank could be established and techniques for genetic improvement including production of elite cow could be promoted. Recently, vitrification has been widely used and is now regarded as a potential alternative to conservative slow freezing. Vitrification has potential advantages over conventional freezing in that it takes only a few seconds for cooling embryos, and there is no extra-cellular crystallization, which is one of the major causes of cell injury (Rall and Fahy, 1985). Especially, vitrification has been successfully applied for cryopreservation of chilling sensitive bovine oocytes and embryos at various developmental stages (Mahmoudzadeh et al., 1993; Otoi et al., 1998; Le Gal and Massip, 1999), and live offsprings have been obtained from vitrified -thawed cattle oocytes (Fuku et al., 1992; Vajta et al., 1998). However, Martino et al. (1996) found that chilling damage occured very quickly for *in vitro* matured bovine oocytes, and the developmental potential was halved by exposure to 0°C for 5 sec. To obtain a more rapid cooling rate (14,000 ~20,000°C/min), some investigators brought oocytes in a minimum volume of vitrification solution into direct contact with LN_2 with use of the support of an electron microscope (EM) grid, open pulled straw or micro-dropping (Martino et al., 1996; Vajta et al., 1998; Papis et al., 1999). They suggested that the developmental capacity could be improved by reducing the time to traverse the damaging temperature. However, almost all of these techniques are based on the direct contact of the LN_2 and the cryoprotectant medium containing the oocytes or embryos, which may be a source of contamination (Tedder et al., 1995; Fountain et al., 1997). These dangers may be eliminated by using sterile LN_2 for cooling, then wrapping the cooled embryos in a hermetic container before storage (Lane et al., 1999). As a possible solution to the problem, in this study we used MVC straw for bovine *in vitro* matured oocytes, where the oocytes are loaded in an extremely low volume (< 2 μl) onto the wall of a 0.25 ml straw, which is sealed before cooling. By MVC method, we obtained higher *in vitro* survival rate (91.2%) of vitrified *in vitro* matured Hanwoo oocytes than that of Martino group using EM-grid (1996, 72%). Also, when the *in vitro* developmental capacity of vitrified Hanwoo *in vitro* matured oocytes by MVC method was examined, there were no significant difference compared to control group or exposed group to

cryoprotectants (27.9% vs 36.3% & 32.3%). In addition, when those *in vitro* developed embryos derived from vitrified oocytes were transferred into recipients, some of them are ongoing into term.

This result demonstrated that MVC method is an appropriate freezing method for the Hanwoo *in vitro* matured oocytes and that ovum bank can be maintained efficiently by MVC cryopreservation method .

V. 요 약

본 연구는 체외에서 성숙된 한우 미수정란이 새로운 초자화 동결 방법인 MVC 방법으로 성공적으로 동결 보존될 수 있는지의 여부를 확인하고자 실시하였다. 초자화 동결을 위해서 미수정 난자는 EG10에서 5~10분간 전처리하고 EG30에서 30초간 노출하였으며 0.25 ml 스트로의 내벽에 난자를 각각 적하한 다음, 곧바로 액체 질소에 침지하였다. 융해는 37℃에서 4단계로 이루어졌다 (1.0M sucrose (S), 0.5 MS, 0.25 MS와 0.125 MS). 한우 미수정 난자를 MVC 방법을 이용하여 초자화 동결하였던 바 체외에서의 발생능이 다른 군과 유의한 차이를 나타내지 않았다 (생존율, 난할율, 배반포 형성율: 동결군 - 91.2, 69.4, 27.9%; 노출군 - 100.0, 74.4, 32.3% ; 대조군 - 100.0, 78.3, 36.3%). 또한, 체외에서 발달된 동결군의 난자를 6마리의 대리모 소에 이식하였던 바, 4마리가 임신에 성공하여 이중 3마리가 임신중임이 임신 250일에 직장검사를 통하여 확인되었다. 따라서, MVC 동결 방법은 한우 미수정란을 동결하기에 적합한 방법이며 앞으로 이 방법을 통하여 난자은행이 효율적으로 이용될 수 있으리라 사료된다.

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