Effects of Vitrification Method on the *In Vitro/In Vivo* Development of Bovine Follicular Oocytes

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Since the first mammalian oocytes were successfully frozen and thawed in mouse and the hamster by using freezing method of zygotes and cleavage-stage embryos (Parkening *et al.*, 1976; Tsunoda *et al.*, 1976), it has known that cryopreservation of follicular oocytes is much more difficult than that of late stage embryos (Fahning and Garcia, 1992). Successful cryopreservation of cattle oocytes would offer many advantages of increasing the efficiency of IVF and facilitating fundamental studies of both cryobiology and fertilization itself. Furthermore, 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.

Cattle oocytes are sensitive to low temperatures, despite the efforts of numerous research groups (Glass & Voelkel, 1990; Lim *et al.*, 1991; Schellander *et al.*, 1994), cryopreservation of bovine oocytes remains a difficult task. Aman and Parks (1994) showed that the metaphase II spindle was damaged in 56% of bovine oocytes cooled to 25℃ for only 1 min, and only 10% of bovine oocytes cooled to 4℃ for 1 min contained normal spindles. The reduction in developmental competency of bovine oocytes is highly correlated with the duration of exposure to 0℃. By earlier slow-freezing procedures, the development rates to blastocysts of frozen-thawed oocytes have been low, usually amounting to <3% of all oocytes cryopreserved (Lim *et al.*, 1991). Only a limited number of publications reported blastocyst and subsequent calf development from cryopreserved oocytes (Fuku *et al.*, 1992; Hamano *et al.*, 1992; Otoi *et al.*, 1992). In recently developed vitrification protocols, the blastocyst rates after fertilization and *in vitro* culture of frozen-thawed bovine oocytes start to approach the values of the non-cryopreserved controls (15%, 25% and 30%; Martino *et al.*, 1996b; Vajta *et al.*, 1998; Papis *et al.*, 1999, respectively) and their transfer resulted in live offspring (Vajta *et al.*, 1998).

At present, vitrification has been widely used and is now regarded as a potential alternative to traditional slow-rate freezing. In conventional freezing method, a balance between the various causes of cryoinjury (i.e., ice crystal formation, toxic and osmotic damage) remains, while in vitrification the main objective is the total elimination of ice crystal injury. When oocytes are cryopreserved by vitrification, ice crystal formation is prevented by use of high concentrations of cryoprotectants (about 5 to 7 M) and high cooling and warming rates. Nonetheless, when oocyte vitrification was carried out by plastic conventional straw, the fastest cooling rate that can be obtained by direct plunging in LN_2 is <2500℃/min (Rall, 1987). Rapid freezing using conventional mini straw exposes oocytes to harmful temperatures (between +15℃ and -15℃) for <1 sec during cooling to -196℃ and again during warming to 35℃. This short exposure might be enough to compromise the viability of some of the

Table 1. Comparison of characteristics between freezing method using three types of oocyte container

Specificity \ Type	Conventional Straw	Electron Microscope (EM) Grid	Minimum Volume Cooling (MVC) Straw
Material	Plastic (0.25 ml mini)	Copper	Plastic (0.25 ml mini)
Freezing solution	EG20/EFS40	EG10/EFS30 or EG10/ES30	EG20/EFS40 or EG20/ES40
Freezing step	Oocyte; 2-step (3 min/1 min)	Oocyte; 2-step (1.5 min/1 min)	Oocyte; 2-step (5 min/1 min)
	Embryo; 2-step (3 min/1 min)	Embryo; 2-step (1.5 min/1 min)	Embryo; 2-step (5 min/1 min)
Pre-freezing	Need	-	-
Warming	Air 10 sec/25℃ water/15 sec.	Directly in 37℃ warming sol	Directly in 37℃ warming sol
Dilution step	Oocyte; 2-step (5 min/each)	Oocyte; 4-step (1 min/each)	Oocyte; 5-step (1 min/each)
	Embryo; 2-step (5 min/each)	Embryo; 4-step (1 min/each)	Embryo; 5-step (1 min/each)
Cooling speed	<2500℃/min	<20000℃/min	<20000℃/min
History	Mamoudzadeh *et al.*, 1993	Martino *et al.*, 1996b	Hamawaki *et al.*, 1999

oocytes. Alternative vitrification methods allowing direct contact between the oocytes containing medium and liquid nitrogen that increased cooling and warming rates (>20000℃/min) have been published using the electron microscope (EM) grids (Martino *et al.*, 1996b; Park *et al.*, 2000), open-pulled straws (Vajta *et al.*, 1998), cryoloops (Lane *et al.*, 1999) or nylon mesh (Matsumoto *et al.*, 2001). Especially, these containers require very small volumes of an oocyte suspension (less than 1~2 μl). In general, cryopreservation protocols involve addition of hypertonic cryoprotective agents (CPAs), cooling, thawing, and removal of CPAs from the cell. All of these procedures have cumulative detrimental effects on cells due to osmotic effects (Oda *et al.*, 1992; Arav *et al.*, 1993), CPA toxicity (Fahy, 1986; Vincent *et al.*, 1990), chilling injury (Aman and Parks, 1994; Martino *et al.*, 1996a), and intracellular ice formation. Although considerable progress has been made in cryopreservation of bovine follicular oocytes, vitrification procedures at the oocyte stage resulted in cell biological alterations in the oocyte after thawing. This result apparently may be reflected in the subsequent fertilization and embryonic development.

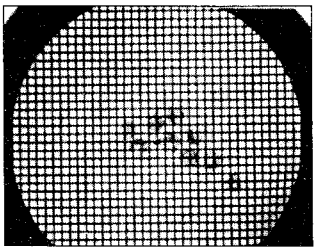
In this study, we tested whether the chilling damage of bovine oocytes can circumvent by three-vitrification method using conventional straw, EM-grid (Kim *et al.*, 1998a,b) or minimum volume cooling (MVC) straw. Vitrification using MVC straw was first described method in bovine blastocyst freezing by Hamawaki *et al.*, (1999). Vitrification using MVC straw has merits that all treatment procedures are very simple, easy, and reliable data can obtain as indicated in previous our report (Kim *et al.*, 2001). Additionally, combination effect between vitrification solutions (EFS or ES) and oocyte containers on bovine oocyte cryopreservation was examined. This study was carried out i) to optimize freezing method for Hanwoo (Korean Native Cattle) follicular oocytes, ii) to examine effect of vitrification and thawing procedures on intracellular microorganelle of bovine oocytes, iii) to confirm *in vivo* developmental capacity after transfer of frozen-thawed, *in vitro* fertilized and developed bovine embryos by the most suitable oocyte vitrification method selected in this study, and iv) to determine whether the proper vitrification method for oocyte stage is also effective to late stage embryos produced from IVF or NT procedures.

A. Conventional straw

CP	4 cm	1.5 cm	0.5 cm	0.5 cm	○ ○ ○ 2 cm	1.0 cm	0.5 MS	SP & HS

Key (left to right); Cotton plug, 0.5 MS, Air, EFS40, Air, EFS40 (oocyte or embryo column), Air, 0.5 MS, Straw powder and heat sealing

B. EM-grid



diameter – 3.05 mm
thick – 0.037 mm

C. MVC straw

CP	Not used	○ ○ ○ ○ ○ ○ oocytes or embryos

Figure 1. Three types of oocyte/embryo container.

Table 2. *In vitro* survival and development of vitrified-thawed bovine GV oocytes

Treatment		No. of oocytes	No. of oocytes survived (%)	No. of ≥2-cell (%)	No. of blastocysts on day 8 (%)	Bla. cell no.* (n=10)
Control		114	-	84 (73.7)	30 (35.7)	121.0±7.0
Freezing	EM-grid/EFS30	139	60 (43.2)[b]	39 (65.0)	12 (30.8)	110.8±8.5
	MVC straw/ES30	180	110 (61.1)[a]	80 (72.7)	31 (38.8)	118.5±5.8

* Blastocysts were examined by Hoechst staining
[a-b] Different superscripts within columns denote significant differences ($p<0.05$)

However, our results demonstrated that Hanwoo follicular oocytes can be successfully cryopreserved not by vitrification method using conventional straw/EFS40, but by vitrification method using either EM-grid/EFS30 or MVC straw/ES30. It means that selection of oocyte container and cryoprotective additives in freezing procedures was very important for the oocyte survival after thawing. Also, it proved that bovine follicular oocytes were highly chilling sensitive, and thus the higher cooling and thawing speed in oocyte cryopreservation is very important. These results were proved by fine results obtained on *in vitro* survival, *in vitro* fertilization and *in vitro* development after thawing. When the freezing effects on intracellular microorganelle and cytoskeletal structure of frozen-thawed oocytes were examined, we confirmed that survived oocytes in MVC straw/ES30 vitrification method were normally developed into embryos. Also, the stability of MVC straw/ES30 vitrification method was proved by *in vivo* developmental capacity and thus four live births were successfully obtained (oocyte stage:

Table 3. *In vitro* survival and development of vitrified-thawed bovine MII oocytes

Treatment		No. of oocytes	No. of survived oocytes (%)	No. (%) of ≥2-cell on day 2	No. (%) of Bla. on day 8	Bla. cell no.* (ICM)
Control		130		102 (78.5)	37 (36.3)	135.0±8.5 (34±7.2)
Freezing	EM-grid/EFS30	275	235 (85.5)	170 (72.3)	56 (32.9)	120.4±6.5 (29±12.0)
	MVC straw/ES30	204	186 (91.2)	139 (74.7)	48 (34.5)	126.8±8.0 (32±10.5)

*Blastocysts were examined by differential staining

Table 4. Pregnancy rates of vitrified Hanwoo IVM 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%
No. of calf	2/3	66.7%

*Day 7 embryos after IVF

two-elite calves, blastocyst stage: two calves). Therefore, this study indicated that by using MVC straw/ES30 vitrification method, cryopreservation of bovine follicular oocyte can be successfully carried out and the establishment of bovine ovum bank can be maintained effectively.

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