韓國家畜繁殖學會誌 23(3) : 221~227(1999)
Korean J. Animal Reprod.

In Vitro Fertilization and Development of Bovine Immature Oocytes by Ultra-Rapid Cooling

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류정순 · 길광수 · 김남형 · 고대환[1] · 이훈택 · 정길생*
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ABSTRACT

Successful cryopreservation of bovine immature oocytes can increase availably of oocytes for the *in vitro* fertilization or nuclear transfer. However, it was not reported successful development to the blastocyst stage following *in vitro* fertilization of cryopreserved bovine immature oocytes. The objective of this study was to determine the incidence of survival, meiotic maturation, fertilization and *in vitro* development of cryopreserved bovine immature by ultra rapid cooling methods. The oocytes were adversely affected by brief exposure to EFS40 solution in electron microscope grids and plunged directly into liquid nitrogen. After such ultra-rapid cooled immature oocytes were warmed, 78% of oocytes were matured to the metaphase Ⅱ stage, 50% of oocytes were fertilized after insemination, and 5% of oocytes were developed to the blastocyst stage. Different sodium concentration of sodium ion in the freezing medium did not affect survival, maturation, fertilization and *in vitro* development of cryopreserved oocytes. These results suggested that immature bovine oocytes can be cryopreserved by ultra-rapid cooling methods.

(Key words : Cryopreservation, Bovine oocytes, *In vitro* fertilization, *In vitro* culture)

Ⅰ. INTRODUCTION

The ultra-rapid cooling method using electron microscope grids has been recently developed to cryopreserve bovine oocytes (Martino et al., 1996). Very high cooling rates achieved by direct plunge into liquid nitrogen might enable the oocytes to pass through the damaging temperature zone in the fluid state and to lead oocytes solidified states below −130℃. Martino et al. (1996) suggested that this simple method was not harmful for the oocyte viability, but the use of inappropriate cryoprotectant could effect the developmental ability of cryopreserved oocytes.

Ethylene glycol is a permeable component and has been widely used for the cryoprotectant during vitrification or ultra-rapid cooling procedure (Matino et al., 1996). Kasai et al. (1990, 1992) have developed a vitrification solution

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called EFS40, which consists of 40% ethylene glycol, 18% ficoll, and 0.3M sucrose dissolved in modified PBS. In mouse and rabbit embryos, very high developmental rates were reported following freezing and thawing *in vitro* preservation solution (Kasai et al., 1990, 1992). EFS has also been used for the cryopreservation of bovine embryos (Mahmoudzadeh et al., 1995).

Sodium salts the major components of cryoprotectant if the excessive amount of intracellular sodium exist, sodium pump removes the sodium from the cell. Sodium diffuses freely into the cell, but the excess is removed by sodium pump. During freezing, the sodium pump may become disabled, which result in increase in the flow of water out of the cell as well as an increase in diffusion of sodium ion into the cell. Very recently, Stachecki et al. (1998) reported that substituting choline for sodium as the major extracellular cation enhances development of cryopreserved unfertilized mouse oocytes following *in vitro* fertilization

Cryopreservation of immature oocyte at the germinal vesicle stage would give the advantages such as to provide readily available source of oocytes for research, and to allow experiments to be performed at the convenient time. However, as compared to mature oocytes, the higher sensitivity of immature oocytes in cool environments has been reported in the cattle (Schllander and Taha, 1992; Le Gal, 1995; Lim et al., 1992). In the mouse, even with limited success rate, development to the blastocyst stage from cryopreserved GV-stage oocytes has been reported.

In the present study we have investigated survival, maturation, fertilization and *in vitro* development of immature bovine oocytes following ultra-rapid cooling using ethylene glycol based solution EM grid. In addition, effect of sodium concentration in the cryoprotectant was also determined.

Ⅱ. MATERIALS AND METHODS

1. *In vitro* Maturation

Bovine cumulus-oocyte complexes (COC) with uniform ooplasm and compact cumulus cells were prepared in HEPES buffered Tyrode,s Albumin Lactate Pyruvate (TALP) medium containing 0.1% polyvinylalcohol. Culture medium for *in vitro* maturation was Tissue Culture Medium 199 (TCM199) supplemented with 10% fetal bovine serum (Gibco BRL, NY1004879), 0.2 mM sodium pyruvate, 1 μg/ml FSH and 1 μg/ml estradiol-17β. Five to ten COC were cultured in 500 μl of maturation medium at 39℃ in an atmosphere of 5% CO_2 in air.

2. Oocyte Cryopreservation

Oocytes were frozen using a procedure based on a method developed for the cryopreservation of bovine oocytes (Martino et al., 1996). Briefly, oocytes in a group of 5 to 10 were exposed to 3 ml of the 5.5M ethylene glycol or EFS40 for 20 seconds. The oocytes were then moved to the electrone microscope copper grids (GQ 400-C, Ted Pella, Inc., Redding, CA). The blotted grids were immediately plunged into liquid nitrogen. In order to thaw the oocytes, the grids with oocytes were transferred to the culture dish containing 4 ml of 0.5 M sucrose (prepared in DPBS containing 10% FBS) at 37℃.

3. Evaluation of Survival

The oocytes were thawed approximately 24 h after cryopreservation. After *in vitro* fertilization, survival of zygote was evaluated based on cytoplasmic appearance and membrane integrity. Cleavage was evaluated at 48 h after insemination. The embryos were then cultured for additionally for 6 days.

4. Assessment of Sperm Penetration, Pronuclear Formation and Development *in vitro*

At 20 h after insemination, oocytes were fixed with 2% formalin solution for 2-3 minutes and stained with bisbenzimide solution (No. 33342, 2.5μg/ml, Sigma) and then examined for nuclear morphology at 200× and 320× magnification with phase contrast microscopy. After 8 days of culture, the percentage of *in vitro* fertilized oocytes developing to the cleavage, morula and blastocyst stages were determined. The number of nuclei in embryos that developed to morula and blastocyst stage was counted after staining with bisbenzimide solution.

5. Experimental Design

Experiment 1. Cryopreservation of immature bovine oocytes

The immature oocytes were cryopreserved using ultra rapid cooling method in the presence of EFS40. The incidences of meiotic maturation, fertilization and development to the blastocyst stage were determined.

Experiment 2. Effect of altering the sodium concentration in the cryoprotectant.

The effect of substituting choline for sodium in freezing medium on immature oocyte cryopreservation was examined. The Dulbecco's phosphate buffered Saline (DPBS) was modified by substituting an equal amounts of NaCl to 0, 68.5 or 137.0 mM choline chloride. The incidence of maturation, fertilization and *in vitro* development rates were determined after cryopreservation of the oocytes.

6. Statistical Analysis

All experiments were replicated at least three times. All results were analyzed by X^2 test.

Ⅲ. RESULTS AND DISCUSSION

Experiment 1. Cryopreservation of immature bovine oocytes

The incidences of survival, meiotic maturation, fertilization and development to the blastocyst stage were determined following thawing of cryopreserved bovine immature oocytes. As shown in Table 1, significant lower incidence of survival of cryopreserved immature oocytes has been observed following thawing. However, the percentage of meiotic maturation among survived oocytes was not different with that of control (Table 1).

The incidences of sperm penetration, polyspermy, pronuclear formation were shown in the Table 2. Higher incidence of polyspermic penetration was observed in the cryopreserved immature bovine oocytes as compared with that in control. However, the percentage of male and female nucleus formation was not different. Lower incidence of development to the blastocyst

Table 1. Meiotic maturation of immature oocytes following ultra rapid freezing mothed

Treatment	Number of oocytes		Number(%) of survived oocytes assessed			
	Examined	Survived(%)	GV	GVBD	M Ⅰ	M Ⅱ
Control	83	83(100)[a]	4(4)	2(2)	5(6)	72(87)
Exposure CPA	85	80(94)[b]	4(5)	0(0)	6(7)	70(88)
Freezing	120	58(48)[b]	1(2)	0(0)	12(21)	45(78)

GV, germinal vesicle; M Ⅰ, metaphase-Ⅰ, M Ⅱ, metaphase-Ⅱ

[a,b] Different superscripts within column denote significant differences ($P<0.05$)

Table 2. Fertilization of cryopreserved immature bovine oocytes at 20 h following insemination

Treatment	Number of oocytes examined	Number (%) of oocytes			No. of sperm in penetrated oocyte (Mean±SEM)
		Penetrated	Polyspermic	Formed male and female PN*	
None	100	95(95)[c]	23(24)[a]	71(75)	1.5±0.4
Exposure CPA**	80	68(85)[d]	28(35)[b]	56(70)	1.6±0.4
Cryopreserved	120	92(77)[d]	38(41)[b]	60(50)	1.8±0.6

[a,b] Different superscripts within column denote significant differences ($P<0.05$)

[c,d] Different superscripts within column denote significant differences ($P<0.05$)

*PN: pronucleus

**CPA: cryoprotectant

Table 3. *In vitro* development of cryopreserved bovine oocytes following in vitro fertilization

Treatment	No. of oocytes	No. of eggs developed to		Cell number of blastocysts
	Used	2-cell	Blastocysts	
Control	100	73(73)[a]	23(23)[a]	128±5
Exposure CPA*	80	56(70)[a]	8(10)[b]	102±7
Freezing	200	89(44.5)[b]	10(5)[b]	98±8

[a,b] Different superscripts within columns denote signification differences ($P<0.05$)

*CPA: cryoprotectant

stage was seen in the cryopreserved bovine immature oocytes following *in vitro* maturation and fertilization. Higher incidence of polyspermy in either freezing or thawed oocytes is probably due to abnormal organization cf cytoskeletal alteration during cryopreservation which results in the appropriate cortical granule exocytosis. Previous study showed that frozen and thawed mouse oocytes have a reduced incidence of fertilization, its probable due to premature release of cortical granules (Vincent et al., 1990). Sxhalkoff et al (1989) also found that exposure of mature mouse and human oocytes to high osmolar cryoprtectants caused the premature cortical granule release. Fuku et al. (1995) reported that both immature or mature oocytes exposed in the cryoprotectant release prematurely cortical granules which possibly induce polyspermy. Effects of exposure to the cryoprotectant or freezing methods on the cortical granule reaction are in progress in our laboratory.

In this study we use EFS40 as the cryoprotectant. EFS40 has been developed for the cryopreservation of mouse oocytes, has also been used for the vitrification of rabbit (Kasai et al., 1992), bovine morula and blastocysts (Mahmoudzadeh et al, 1993; Tachikawa et al., 1993). Successful cryopreservation of mammalian immature oocyte s could increase the efficiency of *in vitro* fertilization using ovum bank, as well as provide fundamental studies of both cryobiology and early embryonic developmen. Ultra-rapid cooling method using electron microscope grids has first been developed for the cryopreservation in drosophila embryos (Steponkus et al., 1990; Mazur et al.1990) and now for the bovine mature oocytes (Matino et al., 1996). Electron microscope grids have been used to support oocytes to plunge into directly into LN2. Very high cooling rates (estimate 3000℃/min) archived

Table 4. Chemical composition of modified PBS medium (g/l)

Composition	Medium 1	Medium 2	Medium 3
NaCl	8.000	4.000	–
KCl	0.200	0.200	0.200
Na_2HPO_4	1.150	1.15.	1.150
KH_2PO_4	0.200	0.200	0.200
$MgCl_26H_2O$	0.100	0.100	0.100
glucose	1.000	1.000	1.000
Choline Cl	–	9.550	19.10
$CaCl_2$	0.100	0.100	0.100
Phenol red	0.005	0.005	0.005

by directly plunging into liquid nitrogen might enable the oocytes to pass through the damaging temperature zone in the fluid state and to lead them solidified states below −130℃.

Experiment 2. Effects of sodium ion in the cryoprotectant

The formulation of cryopreservation medium, containing chloride and no sodium chloride, was shown in Table 4. The incidence of meiotic maturation, fertilization and *in vitro* development was determined when NaCl was replaced choline chrolide. As shown in Tables 5 and 6. there was no significant difference in incidence of maturation, fertilization and cleavage among treatment. Sodium salts are the major components of cell culture media including those used for cryopreservation of mammalian embryos. Sodium ion might contribute significantly to the solution effects during cooling and rewarming of embryos. Stachecki et al. (1998) reported that substituting choline for sodium as the major extracellular cation in the cryopreservation of unfertilized mouse eggs enhanced survival and *in vitro* development rates. In the present study, we did not find any effects of sodium ion the *in vitro* fertilization and *in vitro* development of cryopreserved bovine oocytes. This is probably due to difference of freezing methods. During slow freezing oocytes, the detrimental effect possibly inhibit Na/K pump of oocytes (Stachecki et al., 1998). In the ultra-rapid methods, the sodium ion not have enough time to affect to the cell membrane function such as Na/K pump.

Table 6. Effects of sodium ion on the *in vitro* development of cryopreserved bovine immature oocytes following IVM and IVF

Treatment	No. of oocytes examined	No. of oocytes developed to	
		Cleavage	Blastocysts
Medium 1	50	31(62)	3(6)
Medium 2	50	36(72)	4(8)
Medium 3	50	26(52)	3(6)

Table 5. Effect of sodium ion on the meiotic maturation and fertilization of bovine immature oocytes following ultra rapid freezing and thawing

Treatment	Number of oocytes	%. of survived oocytes			
	Examined	Matured	Penetrated	Polyspermy	With male & female pronucleus
Medium 1	100	70	74	38	54
Medium 2	100	64	80	40	51
Medium 3	100	60	61	28	43

Ⅳ. 요 약

미성숙한 소 난자 동결보존 기술의 개발은 체외수정, 복제동물 및 형질전환동물 생산에 필요로 하는 난자를 시간과 공간의 제약 없이 공급이 가능해지기 때문에 그 효용가치가 많으나 아직까지 성공 보고례가 없다. 본 연구에서는 전자현미경용 grid를 이용한 초급속 동결 방법에 의해 미성숙한 소 난자를 동결 보존한 후, 이 난자를 융해하여 체외성숙, 체외수정 및 배발달을 유도하였다. 동해제는 PBS에 40% ethylene glycol, 0.5M sucrose, 18% ficoll과 10% fetal bovine serum이 들어 있는 EFS40을 사용하였다. 동결·융해 후의 난자의 생존율은 48%정도로 대조군에 비해 현저히 낮았으나 metaphase-Ⅱ까지의 성숙율은 78%로, 정상 자웅전핵 형성율 75%로 대조군에 비해 차이가 없었다. 또한 배반포까지의 배발달율은 대조군 23%와 동결군은 5%로 동결 융해한 것이 낮았으며 수정후 108~120시간 째 배반포를 염색하여 세포수를 알아본 결과는 각각 128±5와 98±8이었다. 항동해제내 Na^+이온의 농도에 따른 미성숙된 난자의 생존율, 성숙, 수정 및 배발달율을 조사하였으나 유의차가 없었다. 이상의 결과는 동결 보존된 미성숙 소 난자가 융해후 체외성숙 및 수정에 의해 배반포까지의 발달이 가능하다는 것을 시사하고 있다.

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(접수일자 : 1999. 7. 12. /채택일자 : 1999. 9. 12.)