J. Emb. Trans. (2009) Vol. 24, No. 2, pp. 121~125

Study on Development of Vitrified Bovine Immature Oocytes Following ICSI

Ji-Hoon Park[1], Man-Hee Rhee[2] and Sang Keun Kim[1,*]

[1]*College of Veterinary Medicine, Chungnam National University, Daejeon 305-764, Korea*
[2]*College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Korea*

ABSTRACT

In the present study, effects of concentration of cryoprotectant solutions on the nuclear maturation of vitrified-thawed bovine oocytes were examined. Also, the developmental capacity of vitrified-thawed immature oocytes following ICSI was investigated. Oocytes were cultured in TCM-199 medium supplemented with 5% FBS at 38℃ in 5% CO_2 and air. The *in vitro* maturation rate of vitrified oocytes was 24.5 ± 4.2%. The *in vitro* maturation rate of vitrified oocytes was lower than that of the control (72.0 ± 3.5%, $p<0.05$). The *in vitro* maturation rate of vitrified-thawed oocytes incubated in TCM-199 medium supplemented with 1.0~5.0 ug CB were 26.7 ± 3.2%, 35.7 ± 3.2%, 54.0 ± 3.0%, 42.5 ± 3.6%, respectively. The *in vitro* maturation rate (57.0 ± 3.0%) of the vitrified-thawed oocytes treated with 3.0 μg CB for 20 min was the highest of all vitrification groups, although the maturation rate were significantly ($p<0.05$) lower than those of fresh oocytes. The *in vitro* maturation rates of the vitrified-thawed (with EDS and EDT) oocytes were 53.8 ± 3.4%, 51.1 ± 3.5%, respectively. This results were lower than the control group (72.0 ± 3.0%). The *in vitro* developmental rates of the vitrified-thawed oocytes following ICSI were 28.6 ± 4.5%, 25.6 ± 4.3%, respectively. This results were lower than the control group (40.0 ± 4.0%).

(Key words : bovine oocytes, vitrification, cytoclacin, ICSI, *in vitro* developmental rates)

INTRODUCTION

Although various species have been experimented using vitrification of oocytes development, the overall efficiency of vitrified immature oocytes development is low. There are still many unresolved problems with current vitrification technology.

Research on cryopreservation about survival of embryos (Schmidt *et al.*, 1993; Leibo, 1993) or oocytes (Suzuki and Nishikata, 1992; Robinski *et al.*, 1991; van Blerkom, 1989) after frozen-thawed have been reported, but there was much difference between the reporters and their results. Embryo vitrification are being conducted, because the embryos are kept in overcooling while preventing water from hydrating and ice crystal formation with the addition of high concentrations of cryoprotectants in vitrification solution (Rall and Fahy, 1985; Kasai *et al.*, 1990; Vaita *et al.*, 1998; Cuello *et al.*, 2004). Rall (1992) and Hamlett *et al.* (1989) reported that embryo cells exposed with cryoprotectants during freezing in MI or MⅡ stages had damage of the spindle fiber and external granule. Mazur (1972) reported that during freezing,d damagin reason that the cells died was because the in the cell had ice crystallization and thawing influence and because of this have reported that reasonable equilibriatitime is needed. Renard *et al.* (1984) reported that freezing with short equilibriatitime was capable of two step freezing with the addition of the non-permeable sucrose. Freezing the earernimmature oocytes at different developmental stages was more appropripindeable sucmature germinal vesicle stage. If oocytes that have high fertilization rates and *in vitro* developmental rates after oocyte vitrification can be preserved, it could be assumed that it will be highly utilized for *in vitro* fertilization and other fields of biotechnology. However, an urgent subject need to be increasement of the survival rates of bovine immature oocytes or embryos are lower than those of experimental animals.

In the present study, effects of cryoprotectant solutions on the developmental rate of vitrified-thawed bovine oocytes were examined. Also, the developmental capacity of vitrified immature oocytes following intracytoplasmic sperm injection (ICSI) was investigated.

MATERIALS AND METHODS

1. Recovery and Culture of Oocytes

Ovaries were collected immediately after slaughter and were

* Correspondence : E-mail : kskkim@cnu.ac.kr

kept at 30℃ saline containing 100 IU/ml, penicillin G and 100 μg/ml streptomycin sulfate. Upon arrival at the laboratory, ovaries were washed three times witn maturation medium. Follicular fluids was collected by 18 G syringe from 2~5 mm follicles. Only cumulus-oocytes complexes (COCs) witn more than two layers of intacllumulusllellsan td witn uni 2~mllytoplasm, were storected 2~5ust. The follicular oocytes culture at t TCM-199 medium supplemented witn 10% FCS, 1 μg/ml FSH, 2 IU/ml18CG, 1 μg/ml β-estradiol, 100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate at 38℃ in 5% CO_2slleubator. All chem wils were purchased from Sigma Chemical Co. (St. Louis, MO U.S.A.) except for those specifically described.

2. Vitrification and Thawing of Oocytes

Before dehydration, the COCs were treated with 1.0, 2.0, 3.0, 5.0 μg cytoclacin B (CB) for 30 min at 38℃. Then vitrification was performed with the use of EDS (20% ethylene glycol + 20% DMSO + 0.5 M sucrose + 10% FCS), EDT (20% ethylene glycol + 20% DMSO + 0.3M trehalose + 10% FCS) + TCM-199 medium. Vitrification immature oocytes are cultured in vitrification solution (VS_1) solution for 1 min afterwards transferred to a 20 ul drop VS_2 solution, and then quickly added to the EDS solution to expose for 1 min. The oocytes were sealed in a 1.0 mm straw and placed in a LN_2 container. Frozen oocytes were rapidly thawed in a water bath at 30~35℃, and then placed in TCM-199 medium containing 0.5 M sucrose and 0.5 M galactose for 5 min each, respectively, at 38℃. After being washed for 2~3 times, using fresh medium the oocytes were cultured in a 10% FCS + TCM-199 medium, The COCs were transferred to a 100 μl droplet of the maturation medium under mineral oil in a Petri dish and cultured at 38℃ under 5% CO_2 in air for 48 hrs.

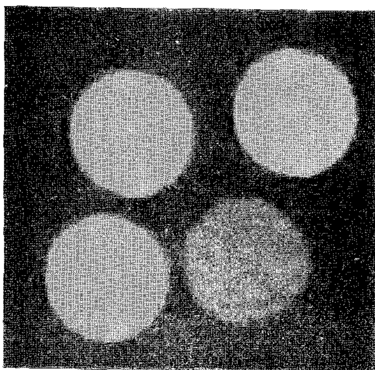

Fig. 1. Morphological appearance of oocytes after vitrification were stained with PI.

3. ICSI Procedure

Frozen semen was rapidly thawed in a water bath at 30℃. The spermatozoa were washed twice Hepes-buffered TCM-199 medium by centrifugation at 800 G for 10 min. The spermatozoa were then exposed to 0.2 uM inophore A23187 for 2 min and resuspended in Hepes-buffered medium supplemented with 3 mg BSA and 1 mM caffeine for 4~6 hrs at 38℃. Five to ten oocytes with a first polar body were loaded into 1.5 ml microcentrifuge tubes containing 500 ul of M_2 medium supplemented with 3 mg/ml BSA and centrifuged at 12,000 g for 3 min to facilitate sperm injection. ICSI was carried out in 2 ul drops of M_2 containing 3 mg BSA. The sperm suspension was placed in droplet of M_2 containing 7% polyvinylpyrrolidone. Each spermatozoon was injected into ooplasm using a micromanipulator (Narishige, Japan) immediately after immobilization.

4. The Assessment of Survival and Developmental Rate

After thawing, the oocytes were denuded of cumulus cells in TCM-199 medium using a pipette by repeated aspiration and expulsion. The cumulus-free oocytes were stained with 20 ug/ml propidium iodide (PI) in PBS containing 0.1% polyvinyl alcohol and incubated for 15 min. The oocytes were examined under ultraviolet light using an epifluorescence microscope (Nikon, Japan) and plasma membrane integrity of oocytes was assessed. The oocytes with disrupted plasma membrane were dyed red with PI. The judgement of oocytes maturation and fertilization *in vitro* was carried out depending on the criteria of maturation by cell and nucleus division and *in vitro* development by investigating embryo development.

5. Statistical Analysis

The results were expressed by treatment as mean ± SD. For comparison of means, Duncan's multiple verification was performed using SAS package of General Lineas's Model (GLM) procedures (SAS Institute, 1996).

RESULT AND DISCUSSION

1. Effect of CB on IVM Rate of Oocytes

The *in vitro* maturation rate of vitrified-thawed immature bovine oocytes and vitrified-thawed oocytes treated with CB are shown in Table 1 and 2.

The *in vitro* maturation rate of vitrified oocytes was 24.5 ± 4.2%. The *in vitro* maturation rate of vitrified oocytes was lower than that of the control (72.0 ± 3.5%, $p<0.05$). The *in vitro* maturation rate of vitrified-thawed oocytes incubated in TCM-199 medium supplemented with 1.0~5.0 ug CB were 26.7 ± 3.2%, 35.7 ± 3.2%, 54.0 ± 3.0%, 42.5 ± 3.6%, respectively. The *in vitro* maturation rate (57.0 ± 3.0%) of the vitrified-thawed oocytes treated with 3.0 μg CB for 20 min was the highest of all vitrification groups, although the maturation rate were significantly ($p<0.05$) lower than those of fresh oocytes.

Table 1. Effects of vitrification on *in vitro* maturation of immature oocytes

Vitrification	No. of oocytes examined	No. of oocytes matured to	
		GV	MⅡ
Control	50	52.0 ± 3.7	72.0 ± 3.5[a]
Vitrified	184	24.5 ± 4.2	42.4 ± 4.5[b]

[a,b] Values within column with different superscript differ ($p<0.05$).

Table 2. Effects of concentration of CB on *in vitro* maturation of vitrified immature oocytes

Vitrification (Concentration of CB)	No. of oocytes examined	No. of oocytes matured to	
		GV	MⅡ
Control	50	52.0 ± 3.7	62.0 ± 3.5[a]
CB 1.0	150	18.0 ± 3.4	26.7 ± 3.2[b]
2.0	152	27.0 ± 3.0	35.7 ± 3.2[b]
3.0	158	34.8 ± 3.2	54.0 ± 3.0[b]
5.0	155	29.7 ± 3.7	42.5 ± 3.6[b]

[a,b] Values within column with different superscript differ ($p<0.05$).

* Oocytes treated with 1.0~5.0 cytoclacin B for 30 min.

2. *In vitro* and Development of Vitrified-thawed Oocytes Following ICSI

In vitro maturation and developmental rates of vitrified-thawed bovine oocytes without distinct differences compared to fresh oocytes following ICSI are shown in Table 3.

The *in vitro* maturation rates of the vitrified-thawed (with EDS and EDT) oocytes were 53.8 ± 3.4%, 51.1 ± 3.5%, respectively. This results were lower than the control group (72.0 ± 3.0%). The *in vitro* developmental rates of the vitrified-thawed oocytes following ICSI were 28.6 ± 4.5%, 25.6 ± 4.3%, respectively. This results were lower than the control group (40.0 ± 4.0%).

DISCUSSION

Freezing the early immature oocytes at different developmental stages was more appropriate than the mature germinal vesicle stage (Rall and Fay, 1985; van der Elst *et al.*, 1993; Candy *et al.*, 1994; Toth *et al.*, 1994). If oocytes that have high fertilization rates and *in vitro* developmental rates after oocyte vitrification can be preserved, it could be assumed that it will be highly utilized for *in vitro* fertilization and other fields of biotechnology. However, an urgent subject need to be increasement of the survival rates of bovine immature oocytes or embryos are lower than those of experimental animals.

The *in vitro* maturation rate of vitrified-thawed immature bovine oocytes and vitrified-thawed oocytes treated with CB are shown in Table 1 and 2. The *in vitro* maturation rate of vitrified oocytes was 24.5 ± 4.2%. The *in vitro* maturation rate of vitrified oocytes was lower than that of the control (72.0 ± 3.5%, $p<0.05$). The *in vitro* maturation rate of vitrified-thawed oocytes incubated in TCM-199 medium supplemented with 1.0~5.0 ug CB were 26.7 ± 3.2%, 35.7 ± 3.2%, 57.0 ± 3.0%,

Table 3. Effects of cryoprotectants on *in vitro* development of vitrified-thawed immature oocytes following ICSI

Vitrification	Cryo protectant	No. of oocytes examined	No. of oocytes matured	No. of oocytes injected	No. of oocytes developed
Control		50	72.0 ± 3.0	64.0 ± 3.8[a]	40.0 ± 4.0[a]
Treatment	EDS	182	53.8 ± 3.4	45.1 ± 3.4	28.6 ± 4.5[b]
	EDT	180	51.1 ± 3.5	40.0 ± 4.6	25.6 ± 4.3[b]

[a,b] Values within column with different superscript differ ($p<0.05$).

46.5 ± 3.6%, respectively (Table 2). The *in vitro* maturation rate (57.0 ± 3.0%) of the vitrified-thawed oocytes treated with 3.0 μg CB for 20 min was the highest of all vitrification groups, although the maturation rate were significantly (p<0.05) lower than those of fresh oocytes. This result was similar than that of Fujihira *et al.* (2004) who reported that the nuclear maturation rate (46.8%) of the vitrified-thawed oocytes treated with 7.5 μg CB for 30 min was significantly higher (p<0.05) than those (13.9~39.2%) of the vitrified-thawed oocytes treated with 0, 2.5, or 5.0 μg CB for 10 or 30 min. From these results, it was shown that a low (<5 μg) CB treatment with a short incubation was not optimal, and treatment with 5.0 μg CB for 30 min would be beneficial for vitrification of oocytes. In vitrification of the oocytes, the factors that influence on the survival are the toxicity of the cryoprotectants, the composition of the vitrification solution and the freezing and thawing speed (Cuello *et al.*, 2004). Vicente and Garcia-Ximenez (1994) reported that rabbit morulae vitrified in 20% ethylene glycol (EG) + 20% Me_2SO solution showed significantly higher blastocyst rates than those in 40% EG alone. However, the maturation rates of oocytes vitrified using EG were significantly higher than those of oocytes vitrified using EG + Me_2SO.

In vitro maturation and developmental rates of vitrified-thawed bovine oocytes without distinct differences compared to fresh oocytes following ICSI are shown in Table 3. The *in vitro* maturation rates of the vitrified-thawed (with EDS and EDT) oocytes were 53.8 ± 3.4%, 51.1 ± 3.5%, respectively. This results were lower than the control group (72.0 ± 3.0%). The *in vitro* developmental rates of the vitrified-thawed oocytes following ICSI were 28.6 ± 4.5%, 25.6 ± 4.3%, respectively. This results were lower than the control group (40.0 ± 4.0%). Fabbri *et al.* (2001) demonstrated that increasing the sucrose concentration from 0.1 M in a freezing medium improved the survival rate of human oocytes after vitrification and prevented the formation of intracellular ice. However, Isachenko *et al.* (1998), who reported the effecwhoflar io reduce injury during the vitrification of immature porcine oocytes, reported that the maturation rate of the vitrified- warmed oocytes treated with 7.5 μg/ml CB for 10~15 min was compared with the non-CB-treated oocytes (22.0% vs. 5.6%). On the other hand, Mullen *et al.* (2004) verified that increased sucrose concentration imposed a greater osmotic stress and increased the likelihood of damage to the spindle. Isachenko *et al.* (1998), who reported that the maturation rate of the vitrified-thawed oocytes treated with 7.5 μg/ ml CB for 10~15 min was compared with the non-CB-treated oocytes (22.0% vs. 5.6%). However, an urgent subject need to be increasement of the survival rates of bovine immature oocytes or embryos are lower than those of experimental animals.

REFERENCES

Candy CJ, Wood M, Whittingham DG, Merriman JA and Choudhury N. 1994. Cryopreservation of immature mouse oocytes. Hum. Reprod. 9:1738-1742.

Cuello C, Sntonia MG, Parrilla I, Tornel J, Vazquez JM, Roca J, Berthelot F, Martinat-Botte F and Martinez EA. 2004. Vitrification of porcine embryos at various development stages using different ultra-rapis cooling procedures. Theriogenology 62:353-361.

Fabbri R, Porcu E, Marsella T, Roccetta G, Venturoli S and Flamigni C. 2001. Human oocytes cryopreservation: New perspectives regarding oocyte survival, Hum. Reprod. 16: 411-416.

Fujihira T, Kishida R and Fukui Y. 2004. Developmental capacity of vitrified immature porcine oocytes following ICSI: Effects of cytochalasin B and cryoprotectants. Cryobiology 49:286-290.

Hamlett DK, Franken DR, Cronje HS and Luus H. 1989. Murine oocyte cryopreservation: Comparison between fertilization success rates of fresh and frozen metaphase Ⅰ and Ⅱ oocytes. Arch. Andol. 23:27.

Isachenko V, Soler C, Isachenko E, Perez-Sanchez F and Grishchenko V. 1998. Vitrification of immature porcine oocytes: effects of lipid droplets, temperature, cytoskeleton, and addition and removal of cryoprotectant. Cryobiology 36: 250-253.

Kasai M, Komi JH, Takakamo A, Tssnoda H. Sakurai T and Machida T. 1990. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. J. Reprod. Fertil. 89:91-97.

Leibo SP and Oda K. 1993. High survival of mouse zygote and embryos cooled rapidly or slowly in ethylene glycol plus polyvinyl pyrrolidone. Cryo-Letters 14:133-144.

Mazur P. 1972. Cryobiology; The Freezing of Biological Systems. Sci. Washington D.C., 168:939-949.

Mullen SF, Agca Y, Broermann DC, Jenkins CL, Johnson CA and Critser JK. 2004. The effect of osmotic stress on the metaphase II spindle of human oocytes, and the relevance to cryopreservation. Hum. Reprod. 19:1148-1154.

Rall WF and Fahy GM. 1985. Ice-free cryopreservation of mouse embryos at −196℃ by vitrification. Nature 313:573-575.

Rall WF. 1992. Cryopreservation of oocytes and embryos : methods and applications. Anim. Reprod. 28:237-245.

Renard JP, Nguyen BX and Garnier V. 1984. Two-step freezing of two-cell rabbit embryos after partial dehydration at room temperature. J. Reprod. Fert. 71:573-580.

Robinski B, Arav A and Devires AL. 1991. Cryopreservation of oocytes using directional cooling and antifreeze glycoproteins. Cryo-Lettters 12:93-106.

Schmidt M, Hyttle P, Grece T and Avery B. 1993. Ultrastructure of frozen thawed bovine *in vitro* matured oocytes. Theriogenology 39:304.

Suzuki T and Nishikata Y. 1992. Fertilization and cleavage of frozen thawed bovine oocytes by one step dilution methos *in vitro*. Theriogenology 37:306.

Toth TL, Jones HW, Baka SC, Muasher S, Veeck LL and Lanzendorf SE. 1994. Fertilization and *in vitro* development of cryopreserved human prophase I oocytes. Fertil. Steril. 61:891-894.

Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen A, Greve T and Callesen H. 1998. Open pulled straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. Mol. Reprod. Dev. 51:53-58.

van Blerkom J. 1989. Maturation at high frequency of germinal vesicle-stage mouse oocyte after cryopreservation: Alterations in cytoplasmic, nuclear, nucleolar and chromosomal structure and organization associated with vitrification. Human Reprod. 4:883-898.

van der Elst JC, Nerinckx SS and van Steirteghem AC. 1993. Slow and ultrarapid freezing of fully grown germinal vesicle-stage mouse oocytes: Optimization of survival rate outweighted by defective blastocyst formation. J. Assist. Reprod. Gene 10:202-212.

Vicente JS and Garcia-Ximenez F. 1994. Osmotic and cryoprotective effects of a mixture of DMSO and ethylene glycol on rabbit morulae. Theriogenology 42:1205-1215.

(접수일: 2009. 5. 14 / 채택일: 2009. 6. 4)