Effects of Cryoprotectants on *In Vitro* Development of Vitrified Immature Porcine Oocytes Following ICSI

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ABSTRACT

In the present study, effects of concentration of cryoprotectant solutions on the nuclear maturation of vitrified-thawed porcine oocytes were examined. Also, the developmental capacity of vitrified-thawed immature porcine oocytes following ICSI was investigated. Oocytes were cultured in NCSU-23 medium supplemented with 5% FBS at 38℃ in 5% CO_2 and air. The *in vitro* maturation rate of vitrified-thawed oocytes (24.1±2.5%) was lower than that of the control (46.0±3.2%, $p<0.05$). The *in vitro* maturation rate of vitrified-thawed oocytes treated with 1.0~5.0 ug CB + NCSU- 23 medium were 22.2±3.0%, 30.7±3.2%, 46.3±3.1%, 38.5±3.2%, respectively. The *in vitro* maturation rate (46.3±3.4%) of the vitrified-thawed oocytes treated with 3.0 μg CB for 30 min was the highest of all vitrification groups, When the *in vitro* developmental rates of the vitrified-thawed (with EDS and EDT) oocytes following ICSI were 18.5±2.5%, 16.4±2.1%, respectively. This results were lower than the control group (24.0±2.5%).

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

INTRODUCTION

Porcine immature oocytes are very sensitive to cooling (Dion *et al.*, 1990), and the *in vitro* maturation rate after vitrification is extremely low (Huang and Holtz, 2002; Isachenko *et al.*, 1998; Kim *et al.*, 2007), and there have been no reports on the development to blastocysts after intracytoplasmic sperm injection (ICSI) followed by *in vitro* culture. And embryos during vitrification (Isachenko *et al.*, 1998; Dobrinsky *et al.*, 2000; Dobrinsky, 2002).

Research on embryo 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. 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. Recently the study of embryo vitrification are being conducted, because the embryos are kept in over-cooling 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 MⅡ stages . Mazur (1972) reported that during freezing, the main reason that the cells died was because the in the cell had ice crystallization and thawing influence. Renard *et al.* (1984) reported that freezing with short equilibrium time was capable of two step freezing with the addition of the non-permeable sucrose. Rall and Fay (1985) reported that freezing the early immature oocytes at different developmental stages was more appropriate than the mature G-V stage (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 porcine immature oocytes or embryos are lower than those of experimental animals and other animals.

In the present study, effects of concentration of CB, and of two types of cryoprotectant solutions on the developmental rate of vitrified-thawed porcine oocytes were examined. Also, the developmental capacity of vitrified immature porcine oocytes following ICSI was investigated.

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MATERIALS AND METHODS

1. Recovery and Culture of Oocytes

Ovaries were collected immediately after slaughter and were kept at 30℃ saline containing 100 IU/ml (Sigma, U.S.A.) penicillin G (Sigma, U.S.A.) and 100 μg/ml streptomycin sulfate (Sigma, U.S.A.). Upon arrival at the laboratory, ovaries were washed three times with maturation medium. Follicular fluids was collected by 18 G syringe from 2~5 mm follicles. Only cumulus-oocytes complexes (COCs) with more than two layers of intact cumulus cells, and with uniform cytoplasm, were selected for use. The follicular oocytes cultured in NCSU-23 medium supplemented with 10% FCS (Sigma, U.S.A.), 1 μg/ml FSH (Sigma, U.S.A.), 2 IU/ml hCG (Sigma, U.S.A.), 1 μg/ml β-estradiol (Sigma, U.S.A.), 100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate were used for the experiment.

2. Vitrification and Thawing of Oocytes

Before dehydration, the COCs were treated with 1.0, 2.0, 3.0, 5.0 μg 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) + NCSU-23 medium (Lim *et al.*, 2005a, b). 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 NCSU-23 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 + NCSU-23 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.

3. ICSI Procedure

Frozen semen was rapidly thawed in a water bath at 30℃. The spermatozoa were washed twice Hepes-buffered NCSU-23 medium by centrifugation at 800 G for 10 min. The spermatozoa were then exposed to 0.2 uM inophore A23187 (Sigma, U.S.A.) for 2 min and resuspended in Hepes-buffered NCSU-23 medium supplemented with 3 mg BSA (Sigma, U.S.A.) and 1 mM caffeine (Sigma, U.S.A.) 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 (Sigma, U.S.A.). Each spermatozoon was injected into ooplasm using a micromanipulator (Narishige, Japan) immediately after immobilization.

4. The Assessment of Developmental Rate

The oocytes were treated with 0.2% hyaluronidase (Sigma, U.S.A.) for 2 min to remove cumulus cells and denuded oocytes were fixed in acetic acid : ethanol (1 : 3) solution for 24 hrs, and then stained using 1% aceto-orcein (Sigma, U.S.A.) or 10 ug bisbenzimide (Hoechst 33342, Sigma, U.S.A.). The judgement of oocytes maturation *in vitro* was carried out depending on the criteria of maturation by cell and nucleus division, and survival rate or *in vitro* development by investigating embryo development and fluorescence diacetate (FDA)-test.

5. Statistical Analysis

The results were expressed by treatment as mean±SD. For comparison of means, Duncans's multiple verification was performed using SAS package of General Linears 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 oocytes are shown in Table 1. The *in vitro* maturation rate of vitrified oocytes was 24.1±2.5%. The *in vitro* maturation rate of vitrified oocytes (24.1±2.5%) was lower than that of the

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

Vitrification	No. of oocytes examined	No. of oocytes matured to	
		GV	MII
Control	50	34.0±4.2	46.0±3.2[a]
Vitrified	54	18.5±2.2	24.1±2.5[b]

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

control (46.0±3.2%, $p<0.05$). The *in vitro* maturation rate of vitrified-thawed oocytes treated with 1.0~5.0 ug CB + NCSU-23 medium were 22.2±3.0%, 30.7±3.2%, 46.3±3.1%, 38.5±3.2%, respectively. The *in vitro* maturation rate (46.3±3.4%) 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).

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

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

The *in vitro* developmental rates of the vitrified-thawed (with EDS and EDT) oocytes following ICSI were 18.5±2.5%, 16.4±2.1%, respectively. This results were lower than the control group (24.0±2.5%). 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 vi

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

Vitrification (Concent. of CB)	No. of oocytes examined	No. of oocytes matured to	
		GV	MII
Control	50	64.0±3.8	46.0±3.2[a]
CB 1.0	54	16.7±2.5	22.2±3.0[b]
2.0	55	21.8±2.7	30.7±3.2[b]
3.0	54	27.8±2.2	46.3±3.4[b]
5.0	52	31.5±3.1	38.5±3.2

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

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

Table 3. Effects of cryoprotectants on *in vitro* development of vitrified-thawed 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±3.2	56.0±2.5[a]	24.0±2.5[b]
Treatment	EDS	54	64.8±2.8	33.3±2.7	18.5±2.5
	EDT	55	59.3±3.0	32.7±2.3	16.4±2.1

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

trification and prevented the formation of intracellular ice. However, Isachenko *et al.* (1998), who reported the effect of CB to 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. 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.

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