

Survival and *In Vitro* Development of Immature Bovine Oocytes Cryopreserved by Vitrification

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ABSTRACT : The present study was undertaken to investigate the effects of PVP concentration and exposure temperature to vitrification solution on the post-thaw survival, *in vitro* maturation and development of immature bovine oocytes (germinal vesicle stage). The vitrification solution (VS) consisted of 40% ethylene glycol (EG)+0.5 M sucrose (S)+10% FBS. PVP was added to VS: 0%, 5% or 10%. The cumulus-oocyte complexes (COCs) were diluted in VS as one step, after 2 min the COCs were loaded in straw and vitrified by direct immersion into liquid nitrogen. For thawing, the straws were plunged into 30°C water bath for 10s. After thawing, the oocytes were diluted in 0.5 M (in DPBS with 10% FBS) sucrose solution for 5 min. The survival rate (FDA-test and trypan blue) of immature bovine oocytes was measured. The survival rate was higher in 5% PVP (91.5%) than in 0% (64.2%) or in 10% PVP (79.7%). The proportion of metaphase II formation was 69.35% in control (no vitrified COCs), 9.3% in 40% EG+0.5 M S+0% PVP and 21.05% in 40% EG+0.5 M S+5% PVP ($p<0.05$). The effect of room temperature (25°C for 10 min) and cold temperature (4°C for 10 min) on COCs were determined in this study. After IVF, the cleavage and blastocysts rate of oocytes exposed to room temperature and cold temperature in VS+5% PVP was significantly different (2 cell: 63.20% vs 37.97%, blastocysts: 18.40% vs 2.53%). The cleavage rates of frozen-thawed oocytes were 20.53% with PVP and 22.13% without PVP ($p>0.05$). Two out of 151 oocytes (1.32%) developed to blastocyst stage after frozen-thawed with 5% PVP ($p>0.05$). Development of oocytes after frozen-thawing to the 2 cell were not significantly affected with or without PVP following IVF. However, the vitrification of immature bovine oocytes with PVP maintained the ability to develop to the blastocyst stage after IVM-IVF and IVC, while no blastocysts were obtained from oocytes vitrified without PVP. These results suggested that PVP has a protective role for vitrification of immature bovine oocytes as far as survival is concerned, however, the protection was not sufficient enough to support blastocyst formation. (*Asian-Aust. J. Anim. Sci.* 2003. Vol 16, No. 1 : 23-28)

Key Words : Bovine Immature Oocytes, Polyvinylpyrrolidone (PVP), Vitrification, Blastocyst

INTRODUCTION

The physical definition of vitrification is the solidification of a solution (glass formation) at low temperatures without ice crystal formation. It is generally thought that these solutions should be better for the preservation of living cells than solutions that crystallize during cooling or warming (Kuleshova et al., 1999). Successful vitrification requires the combination of a high concentration of permeable cryoprotectant and a very rapid cooling rate. However, the high concentrations of cryoprotectant cause cell damage due to the osmotic and cytotoxic effects (Rall and Fahy, 1985; Rall, 1987).

If immature bovine oocytes could be successfully cryopreserved and/or vitrified, the timing of *in vitro*

maturation, *in vitro* fertilization and nuclear transfer will be more manageable. It would also enhance the ability to maintain frozen banks of bovine oocytes for the advancement of genetic improvement and increase availability of materials for basic research.

In earlier studies on bovine oocyte cryopreservation, dimethyl sulfoxide (DMSO), 1,2-propanediol, and glycerol have been used as cryoprotectants (Herrler et al., 1991; Lim et al., 1992; Xu and Betteridge, 1992). Voelkel and Hu (1992) found that ethylene glycol (EG) was an effective cryoprotectant for bovine embryos permitting direct rehydration of thawed embryos. Takagi et al. (1993) suggested that the toxicity of cryoprotectant was related to the exposure time, but EG was relatively nontoxic. *In vitro* matured bovine oocytes have also been shown more permeable to EG, DMSO, 1,2-propanediol than glycerol (Szell et al., 1989). The sucrose, as a non-permeating agent is known to facilitate dehydration and vitrification that further reduced the toxicity of EG by decreasing its concentration (Otoi et al., 1998). The addition of high-molecular weight polymers such as PVP or Ficoll is believed to promote vitrification and may protect the cellular membrane and the zona pellucida from damage during the cooling and warming procedures (Fahy et al.,

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1994). Suzuki and Nishikata (1992) successfully cryopreserved immature bovine oocytes by a combined process of dehydration of the oocyte with sucrose and permeation with 1,2-propanediol before freezing. However, data from immatured bovine oocytes are scarce and the development rates of frozen-thawed oocytes are still low. Suzuki et al. (1996) reported high survival rate and development (blastocysts; 20%) of germinal vesicle (GV) stage bovine oocytes cooled using EG, trehalose and PVP by programmable freezer.

Many problems have been reported in the cryopreservation of the germinal vesicle (GV) and metaphase II stage oocytes. Especially, GV stage oocytes have a relatively little microtubule (MT). Therefore, during the freezing-thawing GV stage oocytes might avoid MT damage. Moreover, oocytes at this stage might be less injured from the osmotic and cytotoxic effects (Ali and Shelton, 1993a,b; Szell and Shelton, 1987). On the contrary, many types of cells are damaged when cooled to temperatures close to 0°C without freezing, called temperature shock or cold shock (Martino et al., 1996).

The present study was carried out to investigate the effects of concentration of PVP and exposure temperature during immersion in vitrification solution on survival of immature bovine oocyte by evaluation of IVM and developmental rates to blastocyst following IVF.

MATERIALS AND METHODS

Collection of oocytes

Ovaries were collected from a local abattoir and brought to the laboratory in physiological saline (0.85%, w/v, NaCl) at 25-30°C within 2 h. The cumulus-oocyte complexes (COCs) in follicular fluid were aspirated from follicles (2-7 mm in diameter) with a 10 ml plastic syringe with an 18-gauge needle. COCs with at least 3-4 layers of cumulus cells and having homogeneous cytoplasm were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS; Gibco, USA) supplemented with 5% fetal bovine serum (FBS; Gibco, USA) and antibiotic-antimycotic (Gibco, USA). The COCs were suspended in TCM199 medium (Earle's salts) with 25 mM HEPES buffer (Gibco, USA) supplemented with 10% FBS and antibiotic-antimycotic. Oocyte selection and manipulation were performed at room temperature.

Vitrification and thawing

The control vitrification solution consisted of 40% ethylene glycol (EG; Sigma, USA), 0.5 M sucrose (S; Sigma, USA) and 10% FBS dissolved in DPBS. PVP (MW 40000, Sigma, USA) was added in 0, 5, and 10% (w/v) in vitrification solution. COCs were immersed directly into vitrification solution (exposed oocytes) or DPBS for 10 min

at 25°C warm plate or 4°C on the ice. After exposure, the COCs were diluted in 0.5 M sucrose (in DPBS with 10% FBS) solution for 5 min. The oocytes were transferred to fresh TCM199 culture medium (with 10% FBS) and washed three times. For vitrification, the COCs were immersed directly into cryopreservation solution for 2 min at 25°C. Following the exposure, 25 to 30 COCs were loaded into 0.25 ml straws and then the straws were directly immersed in liquid nitrogen (frozen-thawed oocytes). The straws were held at -196°C for 1 day to 6 months. For thawing, the cryopreserved straws were plunged into a 30°C water bath for 10s. After thawing, the contents were expelled into a culture dish and the COCs were diluted in 0.5 M sucrose (in DPBS with 10% FBS) solution for 5 min. Oocytes were transferred to fresh TCM199 culture medium (with 10% FBS) and washed three times.

In vitro viability assessments and maturation

For *in vitro* viability assessment of vitrified oocytes, the fluorescein diacetate (FDA; Sigma, USA) test was used. Live oocytes were stained with FDA, while degenerated oocytes were not. The method for FDA test was performed by the procedure of Linda and Trounson (1980). Stock solution was 5 mg FDA/ml acetone and mixed before use by dilution of 1:400,000 in TCM199 without serum. After thawing, 25 to 30 COCs were introduced into the 200 µl droplets TCM199 medium supplemented with 10% FBS, covered with mineral oil in a 35 mm culture dish (Falcon, USA) and cultured for 23 to 24 h at 38.5°C in 5% CO₂ in air. To determine the rate of survival, after 4 to 5 h culture, the cumulus cells surrounding the oocytes were removed by vortexing for 5 min. Oocytes were exposed to FDA solution at 25 to 30°C for 5 min, and transferred to FDA-free trypan blue solution. The survival of stained oocytes was observed under fluorescence microscope using UV light. Oocytes with bright fluorescence were considered to have survived and without fluorescence (trypan blue stain) not to have.

To assess the nuclear maturation after the *in vitro* maturation for 24 h, the oocytes were denuded and fixed with acetic acid 3: ethanol 1 for 24 h and then stained with 1% aceto-orcein. The meiotic stages of fixed oocytes were examined under a phase-contrast microscope to determine the post-thaw maturation rate.

In vitro fertilization

In vitro fertilization was performed using frozen semen and Brackett and Oliphant (BO) medium (Brackett and Oliphant, 1975). Two straws of frozen semen were thawed in a water bath at 37°C for 1 min. To induce capacitating *in vitro*, the semen was washed by centrifugation for 15 min. The sperm pellet was resuspended in the BO medium containing 20 mg/ml bovine serum albumin (BSA; Gibco, USA), heparin (10 µg/ml; Sigma), and 2.5 mM of caffeine

to give a sperm concentration of 1×10^6 /ml. All matured oocytes were transferred into 100 μ l sperm microdroplets under mineral oil for insemination. In addition, unfrozen cumulus-oocyte complexes matured *in vitro* were placed in sperm microdroplets as a control. At 6 h post-insemination, oocytes were transferred into culture medium and washed four times. The cumulus cells surrounding each ovum were removed by pipetting and then cumulus-free oocytes were co-cultured with cumulus cells on the bottom of dishes containing cumulus cell monolayer. The ova were cultured in TCM199 supplemented with 10% FBS at 38.5°C in 5% CO₂ in air.

Embryos in each group were observed under a microscope ($\times 200$, Olympus, Japan) every 24 h following insemination. Development to two cell stage was assessed at 48 h after insemination. The cleaved embryos were cultured for an additional 7 days to evaluate their ability to develop into blastocysts. About one-half of the culture medium was replaced with fresh medium every 48 h after insemination.

Statistical analysis

Data were pooled from 3 to 4 replicates that had been conducted on the same treatment. Statistically significant differences between the groups were analyzed using Duncan test of SAS programming (SAS Institute Inc., USA). Differences where the P value was less than 0.05 were regarded as statistically significant.

RESULTS

Effect of PVP in vitrification solution

Table 1 shows the survival (FDA and trypan blue test) rate of immature bovine oocytes following vitrification in 0, 5, 10% polyvinylpyrrolidone (PVP). The survival (oocytes with bright fluorescence by FDA test) rate was higher in 5% PVP (91.5%) than in 0% (64.2%) and in 10% PVP (79.7%). In this experiment, 5% PVP is effective cryopreservation reagent of the immature bovine oocytes. Most of the oocytes that survived had normal-shaped cytoplasm and zona pellucida. Also degenerated oocytes had nearly indistinguishable cytoplasm and zona pellucida

Table 1. Effect of polyvinylpyrrolidone concentration on the survival rate of immature bovine oocytes following freezing and thawing (evaluated by FDA and trypan blue test)

PVP conc. ¹ (%)	No. of COCs vitrified	No. (%) of COCs	
		Survived	Degenerated
0	81	52 (64.2) ^b	29 (35.8)
5	71	65 (91.5) ^a	6 (8.5)
10	133	106 (79.7) ^{ab}	27 (20.3)

¹The vitrification solution consisted of 40% EG+0.5 M S (sucrose).

^{a,b} Values within columns with different superscripts are significantly different ($p < 0.05$).

from normal oocytes for the naked eyes. The FDA and trypan blue test were applied for classification of degenerated or survived oocytes, in this experiment (Figure 1).

Effect of equilibration temperature

To determine the tolerance to cooling, room temperature (25°C) and low temperature (4°C) exposed oocytes were tested in this study (Table 2). The exposure time was 10 min. The cleavage and blastocyst rates in room temperature control were significantly different from that of low temperature control (2 cell: 78.70% vs 58.97%, blastocysts: 37.04% vs 14.10%) after *in vitro* fertilization. However, with or without PVP, developmental rate in vitrification solution at room temperature was lower than in PBS control ($p < 0.05$; cleavage 78.70% vs 51.89%, 63.20% and blastocysts 37.04% vs 19.81%, 18.40%). Even if there was no significant difference, the difference in cleavage rate on the same temperature was due to the concentration of cryoprotectants. Low temperature and high concentration of cryoprotectants affected the developmental rate of immature bovine oocytes compared with PBS control.

Effects on the nuclear maturation

Table 3 shows the stages of nuclear maturation reached by the oocytes after freezing-thawing and after 24 h of culture. The proportion of metaphase II formation was 21.05% and 9.3% in frozen COCs with 5% PVP and without PVP, respectively, while it was 69.35% in control oocytes ($p < 0.05$).

Cleavage and development rate of vitrified oocytes

As shown in Table 4, 22.13% (40% EG+0.5 M S) and 20.53% (40% EG+0.5 M S+5% PVP) of vitrified COCs

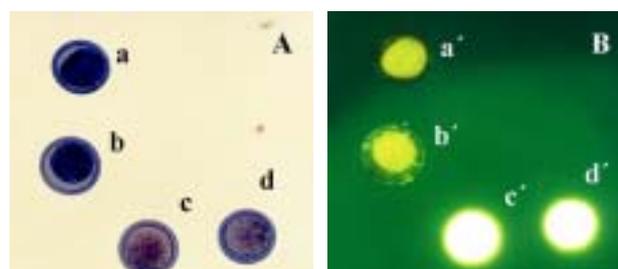


Figure 1. Trypan blue and FDA tested vitrified oocytes. After having been frozen and thawed, oocytes were denuded from cumulus cells and stained in trypan blue and FDA. The survivals were assessed by using fluorescence microscope ($\times 200$). (A) Light microscope. (B) Fluorescence microscope. a, a', b, b': Degenerated oocytes after vitrification at the GV stage (stained for trypan blue and not for FDA). c, c', d, d': Survived oocytes after vitrification at the GV stage (stained for FDA and not for trypan blue).

Table 2. Development after *in vitro* fertilization of immature bovine oocytes exposed at room temperature (25°C) or low temperature (4°C) in vitrification solution

Equilibration temperature	Cryoprotectants ¹	No. of COCs exposed	No. (%) of COCs developed to	
			2 cell	Blastocyst
Room temp. (25 °C)	Control (PBS)	108	85 (78.70) ^a	40 (37.04) ^a
	40% EG+0.5 M S	106	55 (51.89) ^{ab}	21 (19.81) ^b
	40% EG+0.5 M S+5% PVP	125	79 (63.20) ^{ab}	23 (18.40) ^b
Low temp. (4 °C)	Control (PBS)	78	46 (58.97) ^{ab}	11 (14.10) ^b
	40% EG+0.5 M S	85	34 (40.00) ^b	1 (1.17) ^c
	40% EG+0.5 M S+5% PVP	79	30 (37.97) ^b	2 (2.53) ^c

¹EG: Ethylene glycol, S: Sucrose, PVP: Polyvinylpyrrolidone.

^{a,b,c} Values within columns with different superscripts are significantly different (p<0.05).

Table 3. Effect of PVP on the nuclear maturation rate of frozen-thawed immature COCs

Cryoprotectants	No. of COCs vitrified	No. (%) of oocytes developed to ¹					
		GV	GVBD	Met I	Ana I	Tel I	Met II
Control (nonfrozen)	62	3 (4.84)	3 (4.84)	1 (1.61)	3 (4.84)	9 (14.52)	43 (69.35) ^a
40% EG+0.5 M S	86	19 (22.09)	29 (33.72)	25 (29.07)	4 (4.65)	1 (1.16)	8 (9.30) ^b
40% EG+0.5 M S+5% PVP	95	8 (8.42)	22 (23.16)	22 (23.16)	5 (5.26)	18 (18.95)	20 (21.05) ^b

¹Nuclear status; GV: germinal vesicle, GVBD: germinal vesicle breakdown, Met I: metaphase I, Ana I: anaphase I, Tel I: telophase I, Met II: metaphase II.

^{a,b} Values within columns with different superscripts are significantly different (p<0.05).

Table 4. Effects of PVP on the development rate of frozen-thawed immature COCs

Cryoprotectants ¹	No. of COCs vitrified	No. (%) of COCs developed to	
		2 cell	Blastocyst
Control (nonfrozen)	120	97 (80.83) ^a	38 (31.67) ^a
40% EG+0.5 M S	122	27 (22.13) ^b	0 (0) ^b
40% EG+0.5 M S+5% PVP	151	31 (20.53) ^b	2 (1.32) ^b

¹EG: ethylene glycol, S: sucrose, PVP: polyvinylpyrrolidone.

^{a,b} Values within columns with different superscripts are significantly different (p<0.05).

were cleaved at 48 h post-insemination with no significant difference between cryoprotectants. Development of fertilized oocytes after vitrification to the 2 cell and blastocysts were not affected by the cryoprotectants with or without PVP. No blastocysts were obtained from oocytes vitrified without PVP, and only 1.32% were obtained from oocytes vitrified with 5% PVP.

DISCUSSION

The PVP is a macromolecular reagent that reduces the formation of ice crystal so that the cell membrane and zona of oocytes are protected during the freezing and warming (Fahy et al., 1994). Otoi et al. (1998) reported that 10% of *in vitro* matured bovine oocytes cryopreserved without PVP by simple vitrification method developed to blastocysts in which were vitrified in 40% EG with 0.5 M sucrose and three step dilution of cryoprotectants. However, we used immature bovine oocytes, and one step dilution of cryoprotectant in 40% EG plus 0.5 M sucrose with PVP solution for 5 min. In this study, 5% PVP was the optimal concentration for immature bovine oocytes, however, only

1.32% oocytes developed into blastocysts (Tables 1 and 3).

Because bovine oocytes are sensitive to chilling when cooled to subphysiological temperatures, their successful cryopreservation is limited. In this study, injury to GV oocytes occurred after a short exposure to 4°C and resulted in reduction of cleavage rates (Table 2). Various reports (Poolard and Leibo, 1994; Arav et al., 1996; Martiano et al., 1996) showed that a significant reduction in cleavage rate is evident following an exposure of oocytes to 23°C or below. The results are different from other work (Le Gal and Massip, 1999), in that the maturation rate of oocytes exposed to cryoprotectants before maturation was not significantly difference from that of unexposed control oocytes in three step exposure to vitrified solution. In this study, one step dilution may affect in the development of immature oocytes.

The maturation rate (Table 3, 21.05%) of immature oocytes after frozen-thawing in this study was close to the reported by Lim et al. (1992, 27.7%). The low maturation rate of frozen immature oocytes in this experiment demonstrates that GV stage oocytes are very sensitive to vitrification. Most cumulus cells (CCs) surrounding vitrified GV oocytes had numerous cytoplasmic vacuoles, which appeared to disrupt the integration of their cytoplasm during freezing (Fuku et al., 1995). Moreover, Fuku et al. (1995) reported that most cumulus cells (CCs) projections might control the intercellular communication between CCs and oocytes during oocyte maturation. In addition to CCs and oocyte communication damage, vitrified GV stage oocytes were injured in microvilli, mitochondria, cortical granules, vesicles and zona pellucida (Fuku et al., 1995). Most oocytes that survived in FDA analysis did not resume

meiosis in this experiment (Tables 1 and 3), suggesting that the FDA test is an index for survival of oocytes but not for developmental potential of oocytes. Dhali et al. (2000) reported that *in vitro* maturation rate of vitrified-warmed oocytes was 31.5% in buffalo oocytes. The fact that cumulus cells remained functional after a freezing-thawing cycle was already reported for rat (Pellicer et al., 1988) and pig oocytes (Didion, 1990). The low maturation rate of frozen oocytes indicated that bovine immature oocytes were very sensitive to freezing even in the GV stage. It thus proves that the detrimental effects of the freezing procedure directly affected the oocytes and surrounding CCs.

Even though development of frozen-thawed immature oocytes following fertilization to 2 cell and blastocysts were not affected by the presence of PVP, with 5% PVP and pre-freeze exposure (room temp.) there resulted an improved rate of development to the blastocyst stage *in vitro* (Table 4). Martino et al. (1996) using a cryoprotectant solution of 5.5M EG +1M sucrose and a total exposure time of 30s, obtained 15% blastocyst rates when oocytes vitrification was done at very high cooling rate on electron microscope grids, whereas blastocyst rates of less than 1 % were obtained in the vitrification using a 0.25 ml straws. Vajta et al. (1998) and Le Gal and Massip (1999) reported that with open pulled straw (OPS), cooling rate is 7 to 10 times more rapid than with 0.25 ml straws, suggests that cooling rate in the straws may affect the developmental rate after thawing.

The present investigation indicates that the concentration of the cryoprotectants and the equilibration temperature during vitrification both affect the survival, maturation and development of immature bovine oocytes. Vitrification solution containing 40% EG+0.5 M S+5% PVP can be used for vitrification of bovine immature oocytes to maintain their ability to develop to the blastocyst stage after thawing and IVM-IVF-IVC. However, further experiments are needed to clarify the effects of equilibration, cooling rates and times of cryoprotectant removal on developmental capacity of oocytes after vitrification.

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