Developmental Competence of Porcine NT Embryos Constructed by Microinjection of Fibroblast Cells into Vitrified Porcine Oocytes

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ABSTRACT

This study was conducted to investigate the efficacy of vitrification procedure for the cryopreservation of porcine oocytes and the utilization of vitrified oocytes as recipient cytoplasts for somatic cell nuclear transfer (NT), and observed that porcine oocytes are evaluated by pronuclear formation, and parthenogenetic development. Single fetal donor cells were deposited into the perivitelline space of vitrified enucleation oocytes, followed by electrical fusion and activation. NT embryos were cultured in NCSU-23 medium supplemented with 5% FBS, at 38.5℃ in 5% CO_2 and air.

1. When the developmental rates of the oocytes after being culture for 0~10 hours vitrified with EDS and ETS were 42.0%, 38.0%, respectively. This results were lower than the control group(62.2%).
2. When the developmental rates of the oocytes after being culture for 0~10 hours vitrified-thawed with sucrose and glucose, 5% PVP, NCSU-23 supplemented with 10% FBS were 33.3%, 25.9%, respectively. This results were lower than the control group(55.6%).
3. The fusion and development to the blastocyst stage between the NT embryos constructed with the vitrified and non-vitrified oocytes were significant differences. Developmental rate of oocytes and NT embryos constructed with the vitrified or non-vitrified oocytes were 13.0±2.4% and 23.2±2.4%, respectively.

(Key words : vitrification of oocytes, NT, oocytes, cleavage, development rate)

INTRODUCTION

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 (Kasai *et al.*, 1990; Vaita *et al.*, 1998; Cuello *et al.*, 2004).

The rapid development of reproductive technologies, such as *in vivo* maturation and *in vitro* embryo production has lead to increased demand for oocytes. Therefore, it would be a significant advance for basic research and commercial applications (Schroeder *et al.*, 1990 and Candy *et al.*, 1994), if immature oocytes could be cryopreserved before maturation and use. Cryopreservation of immature oocytes has been reported with varying degrees of success in several mammalian species, such as mice (Eroglu *et al.*, 1998 and Moffa *et al.*, 2002), cattle (Suzuki *et al.*, 1996), buffalo (Wani *et al.*, 2004) and humans (Tucker *et al.*, 1998 and Isachenko *et al.*, 2006). However, immature porcine oocytes remain one of the most difficult to successfully cryopreserve due to their sensitivity to cooling and freezing. Many studies have showed that the *in vitro* maturation rate after vitrification is extremely low (Park *et al.*, 2005, Rojas *et al.*, 2004 and Hara *et al.*, 2005). Little success has been achieved with cryopreserving immature porcine oocytes. Isachenko *et al.* (1998) obtained 22% metaphase II (MII) stage oocytes after vitrification of immature (GV) oocytes by pretreating with cytochalasin B (CB). Fujihira *et al.* (2004) improved the maturation rate to 37.1% after vitrification of GV oocytes using a cryotop sheet. The fusion and development to the blastocyst stage between the NT embryos constructed with the vitrified oocytes were no significant differences, and those constructed with non-vitrified control oocytes. For successful reprogramming of the donor nucleus, it must be in G_0 or G_1 when transferred to metaphase II arrested oocytes with greater amounts of maturation promoting factors. This strict synchrony will allow chromosomes to condense properly and will enhance the correct ploidy in the resulting embryos (Quan *et al.*, 2005). 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

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the formation of intracellular ice. 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. Vajta *et al.* (1998) and Kasai *et al.* (1990) who reported that development and cleavage rate of mouse embryos when vitrification-thawed using EFS and EPS were 85.0~95.0%, 80~85% and 90.0~95.0 %, respectively.

The purpose of this study was to investigate the effects of glucose, sucrose on the post-thaw maturation rates of oocytes and evaluate sugars as potential cryoprotectants for vitrification of immature oocytes. and development of NT embryos reconstructed by microinjection of fibroblast cells into porcine oocytes.

MATERIALS AND METHODS

1. Preparation of Oocytes

Oocytes collected from slaughterhouse-derived ovaries were cultured *in vitro* for 40~44 hours at 38℃ under a humidified atmosphere of 5% CO_2 in air in NCSU-23 medium supplemented with 10% FBS (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 for 40~44 hours in an incubator (38℃, 5% CO_2 in air).

2. Vitrification and Thawing of Oocytes

Cumulus-free oocytes at the MⅡ stage were vitrified using vitrification procedures described by Atabay *et al.* (2004). The oocytes were exposed to a medium consisting of 5% ethylene glycol (Sigma, USA) in NCSU-23 supplemented with 10% FCS and 50 ug/ml of gentamycin sulfate for 15 min. They were then immersed in a EDS (vitrified 40% ethylene glycol + 20% DMSO + 0.4M sucrose). ETS (vitrified 20% EG + 20% DMSO + 0.3M trehalose), 5% PVP in NCSU-23 supplemented with 10% FCS and 50 ug/ml of gentamycin sulfate. The oocytes were sealed in a 1.0 mm straw (Vajta *et al.*, 1998a) and placed in a LN_2 container. Frozen Oocytes were rapidly thawed in a water bath at 30℃, and then placed in 0.5 M sucrose for 5 min each. After being washed for 2~3 times, using fresh medium the oocytes were cultured in a 10% FBS + TCM-199 medium and stored in liquid nitrogen for 10 days. Survival of oocytes was morphologically evaluated after 2 h of culture in NCSU-23 medium supplemented with 5% FBS, 10 IU/ml hCG and 10 ng/ml EGF.

3. Preparation of Donor Cell

Vitrified and non-vitrified oocytes with 1st polar body were enucleated mechanically by removing the 1st polar body and approximately 20% of the adjacent cytoplasm with a micropipette of manipulator. Enucleation was carried out under an inverted microscope(Nikon Co., Japan) with manipulators (Narishige Co., Japan) in NCSU-23 medium supplemented with 7.5 g/ml of cytochalacin B (Sigma, U.S.A.) and 10% FBS. Enucleated oocytes awaiting nuclear transfer were cultured in a CO_2 incubator in medium supplemented with 4 mg/ml BSA and 0.1 mg/ml cystine. The head of the fetus was removed using iris scissors, and the brain, four limbs, and soft tissues such as liver and intestines were also discarded by scooping out with two watchmaker's forceps. After twice washing with DPBS, the carcass was minced with a surgical blade on a 100 mm culture dish. Cells were dissociated using DMEM supplemented with 0.1% (w/v) trypsin and 1 mM EDTA for 1~2 h at 39℃. And the suspension was centrifuged at 500 × g for 5 min and subsequently seeded into culture dishes. The cell pellet was resuspended and cultured for 6~8 days in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin G (75 μg /ml), streptomycin (50 μg/ml), 1 mM sodium pyruvate, 1% (v/v) nonessential amino acids at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cells or explants, attached cells were further cultured until confluent, subcultured (1:4 dilution) at intervals of 4 days by trypsinization for 5 min using 0.1% trypsin and 1 mM EDTA and were stored in freezing medium in liquid nitrogen at −196 ℃. Frozen media consisted of 80% (v/v) DMEM, 10% (v/v) DMSO and 10% (v/v) FBS. Before nuclear transfer, frozen cells intended for use as donor cells were thawed, cultured in serum starved DMEM supplemented with 0.5% FBS for 3 days until 80% confluency. And cells for nuclear transfer were retrieved from the monolayer by trypsinization for 30s.

4. Nuclear Transfer and Culture

After 44 h IVM, a denuded oocyte with the first polar body was held with a holding micropipette and the zona pellucida was partially dissected with a fine glass needle to make a slit near the first polar body. The first polar body and adjacent cytoplasm presumably containing the MⅡ chromosomes were

enucleated by a micropipette (30 μm in diameter) in HEPES-buffered NCSU-23 supplemented with 4 mg/ml bovine serum albumin (BSA) and 7.5 μg/mL cytochalasin B (Sigma, USA) at 38℃. Round glossy cells were chosen as donor cells and transferred into the perivitelline space of the enucleated recipient oocytes through the hole made at enucleation. After enucleation, oocytes were stained with 5 μg/mL bisbenzimide (Hoechst 33342, Sigma, USA) for 5 min and observed under a with fluorescence microscope. Oocytes still containing DNA materials were excluded from experiments. Couplets were equilibrated with 0.3 M mannitol solution containing 0.5 mM HEPES, 1.0 mM $CaCl_2 \cdot 2H_2O$ and 0.1 mM $MgSO_4$ for 4 min and transferred to a chamber containing two electrodes that were overlaid with fusion and activation solution. And these Couplets were fused and activated simultaneously with a single direct current pulse of 2.1 kV/cm for 30 μs using an Electro-Cell Manipulator (BTX, Inc., San Diego, U.S.A.). Nuclear transferred embryos were treated with 5 g/ml ionomycin, activated with 1.5 mM 6-DMAP for 4 hr, cultured in NCSU-23 or TL-Heaps medium. After microinjection, reconstructed embryos were placed in Ca free NCSU-23 supplemented with 4 mg/ml BSA and 0.1 mg/ml cysteine at 39℃ in a humidified atmosphere containing 5% CO_2 in air for 30 min. The reconstructed embryos were cultured for 7 days after activation. Stained with 1% aceto-orcein after fixing with methanol : acetic acid (3:1), and microscopically examined (× 200) to assess developmental competence and stage using procedures of Kim *et al.* (1988).

5. Assessment of Maturation and Development

Oocytes were fixed in acetic acid : ethanol (1:3) solution for 24 h then stained using with 1% aceto- orcein (Sigma, U.S.A.) or 10 ug/ml bisbenzimide (Hoechst 33342, Sigma, U.S.A.) and observed under an epifluorescence microscope. 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.

6. Statistical Analysis

All data were subjected to a Generalized Linear Model procedure (PROC-GLM) of the statistical analysis system (SAS Institure, Gary, NC, USA). Differences among treatment means were determined using Duncan's multiple range test and *t*-test. All the data were expressed as least square (LS) mean±S.D. Differences among treatment effects were considered at $p<0.05$.

RESULTS AND DISCUSSION

1. Developmental Rate of Vitrified Oocytes

In vitro developmental rates of oocytes vitrified with EDS and EDT, 5% PVP, NCSU 23 supplemented with 10% FBS are shown on Table 1.

When the developmental rates of the oocytes after being culture for 0～10 hours vitrified with EDS and ETS were 42.0%, 38.0%, respectively. This results were lower than the control group (62.2%). This result was significantly lower than that of Vajta *et al.* (1998) and Kasai *et al.* (1990) who reported that development and cleavage rate of mouse embryos when vitrification-thawed using EFS and EPS were 85.0～95.0%, 80～85% and 90.0～95.0 %, respectively. This result was significantly lower than that of Vajta *et al.* 1998) and Kasai *et al.* (1990) who reported that the highest of added sucrose groups and higher than that reported by Rojas *et al.* (2004) using the same vitrification solution.

2. Developmental Rate of Vitrified-thawed Oocytes

In vitro developmental rates of oocytes vitrified-thawed with sucrose and glucose, 5% PVP, NCSU 23 supplemented with 10% FBS are shown on Table 2.

When the developmental rates of the oocytes after being culture for 0～10 hours vitrified-thawed with sucrose and glucose, 5% PVP, NCSU-23 supplemented with 10% FBS were 33.3%, 25.9%, respectively. This results were lower than the control group (55.6%). An appropriate osmotic stress in the vitrification

Table 1. *In vitro* developmental rate of oocytes by vitrification methods

Treatment of oocytes	No. of oocytes vitrified	No. of oocytes survived	
		Normal(%)	Degenerate(%)
Control	45	28 (62.2)[a]	17 (37.8)
EDS	50	21 (42.0)[b]	29 (58.0)
ETS	50	19 (38.0)[b]	31 (62.0)

* Values with different subscripts in same column were denoted significantly different($p<0.05$)

** EDS(Vitrified 40% ethylene glycol + 20% DMSO + 0.4M sucrose). ETS(Vitrified 20% EG + 20% DMSO + 0.3M trehalose).

Table 2. *In vitro* maturation rates of vitrified-thawed immature oocytes in different thawing methods

Treatment of oocytes	Concentration of supplementation(M)	No. of oocytes examined	Status of oocytes		
			GV	MⅡ	Deg.
Control	1.5	45	8 (17.8)	25 (55.6)[a]	12 (26.7)
Sucrose	0.1	54	15 (27.8)	18 (33.3)[b]	21 (38.9)
Glucose	0.5	54	14 (25.9)	14 (25.9)[b]	26 (48.1)

* Values with different subscripts in same column were denoted significantly different ($p<0.05$)

solution is an important factor for improving the maturation rate of vitrified-thawed immature oocytes, which could be regulated by sugars. 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. 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.

3. Developmental Rate of NT Embryos Constructed with Vitrified or Non-vitrified Oocytes

The fusion and development to the blastocyst stage between the NT embryos constructed with the vitrified oocytes are shown in Table 3.

The fusion and development to the blastocyst stage between the NT embryos constructed with the vitrified and non-vitrified oocytes were significant differences. Developmental rate of oocytes and NT embryos constructed with the vitrified or non-vitrified oocytes were 13.0±2.4% and 23.2±2.4%, respectively. The mean cell number in the blastocysts derived from vitrified oocytes was lower than that of the non-vitrified control ($p<$ 0.05). No significant difference were observed in the rate of denucleation between vitrified (64.3±2.2%) and non-vitrified oocytes (65.5±2.1%).

Table 3. Developmental rate of oocytes and NT embryos constructed with the vitrified or non-vitrified oocytes

Treatment of oocytes	No. of oocytes examined	No. of embryos fused	No. of development to (%)	
			2 cell	BL
Vitrified	55	58.0±3.6	34.0±2.4	13.0±2.4[a]
Non-vitrified	56	62.0±3.1	56.0±3.2	23.2±2.4[b]

* Values with different subscripts in same column were denoted significantly different ($p<0.05$).

No reduction in the enucleation rate was observed in the present vitrified bovine oocytes, which is consistent with the results of (Dinnyés *et al.* 2000). The paper of enucleation of vitrified porcine oocytes were can not find. The difference in the enucleation rate between the present study and that obtained in the frozen oocytes using conventional controlled-rate freezing procedure (Kubota *et al.*, 1998) might be due to differences in the cooling and warming rates.

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(접수일: 2007. 12. 17 / 채택일: 2007. 12. 25)