# Effects of Cryoprotectant, Warming Solution and Removal of Lipid on Viability of Porcine Nuclear Transfer Embryos Vitrified by Open Pulled Straw Method

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## ABSTRACT

**This study was carried out to investigate the effects of cryoprotectants, warming solution and removal of lipid on open pulled straw vitrification (OPS) method of porcine embryos produced by nuclear transfer (NT) of fetal fibroblasts. All solutions used during vitrification were prepared with holding medium consisting of 25 mM Hepes buffered TCM199 medium containing 20% fetal bovine serum (FBS) at 38.5℃. The blastocysts derived from NT with or without lipid were vitrified in each medium of different concentrations of dimethyl sulfoxide (DMSO) and ethylene glycol (EG). Also, blastocysts after cryopreservation were warmed into different concentrations of sucrose in warming solution. The optimal concentrations of cryoprotectants in vitrification solution were 10% DMSO + 10% EG in vitrification solution 1 (VS1) and 20% DMSO + 20% EG in vitrification solution 2 (VS2). The optimal concentrations of sucrose were 0.3 M sucrose in warming solution 1 (WS1) and 0.15 M sucrose in warming solution 2 (WS2). Lipid removal from oocytes before NT enhanced the viability of NT embryos after vitrification. Our results show that use of the OPS method in conjunction with lipid removal provides effective cryopreservation of porcine nuclear transfer embryos.**

**(Key words : Vitrification, Cryoprotectants, Warming solution, Lipid removal, Porcine nuclear transfer blastocyst)**

## INTRODUCTION

Reliable cryopreservation of embryos is an important tool for maximizing genetic improvement in domestic species. It also provides low cost import and export of genetic material with minimum risk of disease transmission. Various methods for embryo cryopreservation, such as conventional slow freezing, vitrification and open pulled straw vitrification (OPS), have been successfully used in a variety of species including mouse, cattle, rabbit and horse embryos (Isachenko *et al.*, 2003; Albarracin *et al.*, 2005; Isachenko *et al.*, 2005; Moussa *et al.*, 2005; Naik *et al.*, 2005).

Development of OPS technology (Vajta *et al.*, 1997), which overcomes the drawbacks of traditional vitrification by accelerating the rates of cooling and warming, was reported to be successful with all development stages of *in vitro* fertilized bovine embryos and *in vitro* matured bovine oocytes (Vajta *et al.*, 1998). However, porcine embryos are known to be more sensitive to damage caused by cryopreservation than other mammal embryos are (Pollard and Leibo, 1994). Pig embryos suffer from sensitivity to hypothermic conditions, which limit their ability to be cryopreserved by conventional slow freezing method (Wilmut, 1972). The presence of a large number of intracytoplasmic lipid droplets has been mentioned as an obstacle to successful freezing of porcine embryos. Nagashima *et al.* (1994, 1995) showed that removal of cytoplasmic lipid droplets in embryos enhanced the tolerance of porcine embryos to chilling and that remarkably improved embryos viability following cryopreservation. The transfer of delipidized porcine morulae and blastocysts was demonstrated to provide a practically feasible birth rate after cryopreservation (Dobrinsky, 2002).

These advancements in cryopreservation of porcine embryos have been successful only for *in vivo*-derived embryos, whereas in recent pig cloning research, an *in vitro* matured (IVM) oocyte is usually employed (Betthauser *et al.*, 2000; Polejaeva *et al.*, 2000; Boquest *et al.*, 2002; Hoshino *et al.*, 2005). Most recently, *in vivo*

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derived porcine embryos have been successfully cryopreserved by several groups (Dobrinsky *et al.*, 2000; Beebe *et al.*, 2002; Misumi *et al.*, 2003). Cryopreservation of porcine embryos produced *in vitro* maturation, fertilization and culture remains a challenge due to the limited information on cryopreservation. Recently improved survival rates after cryopreservation of *in vitro* fertilized embryos are attributed to the improved culture conditions (Men *et al.*, 2005). The embryos produced *in vitro* by nuclear transfer (NT) were expected to be even more difficult to successfully cryopreserve than *in vivo* produced porcine embryos.

The quality of porcine oocytes is different with season. Cryopreservation will facilitate the long term storage of porcine cloned embryos with good quality. The current experiment was designed to investigate the effects of cryoprotectants, warming solution and removal of lipid on cryopreservation of porcine embryos produced by NT of fetal fibroblasts.

## MATERIALS AND METHODS

### Collection and *In Vitro* Maturation of Porcine Oocytes

Ovaries were collected from prepubertal gilts at a local slaughter house and transported to the laboratory in 0.9% saline at 30~35℃. Follicular fluid and cumulus-oocyte complexes (COCs) were aspirated from follicles of 2~6 mm in diameter using an 18-gauge needle fixed to a 10 ml disposable syringe. The follicular contents were pooled into 50 ml tube and allowed to sediment, and the sediment was placed into Hepes buffered Tyrode-Lactate medium (TL-Hepes-PVA) containing 0.1% polyvinyl alcohol (PVA). Oocytes with uniform ooplasm surrounded by a compact cumulus cell mass were selected and washed with TL-Hepes-PVA and then washed twice with the maturation medium. The basic media used for *in vitro* maturation with modified tissue culture medium (TCM)199 supplemented with 26.19 mM sodium bicarbonate, 3.05 mM glucose, 0.91 mM sodium pyruvate, 75 μg/ml sodium penicillin G, 50 μg/ml streptomycin sulfate and 0.1% PVA. COCs were cultured in maturation medium containing the 0.5 μg/ml LH, 0.5 μg/ml FSH, 10 ng/ml epidermal growth factor (EGF), 10% porcine follicular fluids (pFF) and 0.57 mM cysteine in the presence of 2 follicle shells. After 22 hr of culture, oocytes were cultured without hormones for 22 hr at 38.5℃, 5% $CO_2$ in air.

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### Preparation of Porcine Fetal Fibroblasts

Porcine fetal fibroblast cells were obtained from fetus on day 35 of gestation. After the brain, intestines and four limbs were removed, tissue was cut into small pieces with fine scissors. Cells were incubated for 30 min at 38.5℃ in PBS containing 0.05% trypsin and 0.5 mM EDTA, and the suspension was centrifuged at 500 × g for 20 min. The cell pellets were resuspended and cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 75 μg/ml sodium penicillin G and 50 μg/ml streptomycin sulfate. The cells were passaged two times, and then frozen using DMEM supplemented with 10% dimethyl sulfoxide (DMSO). To be used as donor cell in nuclear transfer, porcine fetal fibroblast cells were thawed and cultured until they reached confluence. Before nuclear transfer, cells were treated with 0.05% trypsin-0.5 mM EDTA for single-cell isolation at 2~5 min in 38.5℃ incubator. After washing with PBS, cells were resuspended in manipulation medium consisting of TCM199 supplemented with 0.6 mM $NaHCO_3$, 3.15 mM Hepes, 30 mM NaCl, 60 μg/ml sodium penicillin G, 50 μg/ml streptomycin sulfate and 0.3% bovine serum albumin (BSA).

### Lipid Removal of Porcine Oocyte

After removal of cumulus cells, oocytes were then centrifuged in manipulation medium at 10,000 × g for 5 min. The lipid droplet was polarized and removed by the same glass pipette of enucleation in an oocyte (Nagashima *et al.*, 1994; Li *et al.*, 2006).

### Nuclear Transfer of Fetal Fibroblast and *In Vitro* Culture of Porcine Oocytes

After a total of 44 hr of maturation, the cumulus cells were removed from the oocytes by pipetting in manipulation medium supplemented with 0.1% hyaluronidase. For micromanipulation, oocytes and donor cells were placed in a 50 μl drop under oil of manipulation medium supplemented with 7.5 μg/ml cytochalasin B. Oocytes were enucleated by removing the first polar body along with adjacent cytoplasm containing the metaphase plate using a glass micropipette with an inner diameter of 20 μm. Through the same hole in the zona pellucida created during enucleation, a small cell (15~20 μm in diameter) was then placed in contact with the cytoplasm of each oocyte to form a couplet. After manipulation, couplets were washed once and equilibrated in TCM199 for 2 hr at 38.5℃, 5% $CO_2$ in air before fusion and activation. Fusion/activation was accomplished with 1 DC pulse of 1.2 kV/ cm for 30 μsec provided by a BTX Electro-cell Manipulator 200 (BTX, San Diego, CA, USA). Fusion medium was 0.3 M mannitol supplemented with 1.0 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$ and 0.5 mM Hepes. The reconstructed embryos were immediately cultured in PZM-3 medium containing 0.3% BSA at 38.5℃, 5% $CO_2$ in air for 5~6 days.

### Evaluation of Blastocyst Produced *In Vitro*

Blastocysts were observed under stereo-microscope to determine survival, degeneration and formation of expanded blastocysts. To count cell number, all blastocysts were fixed with 2% formaldehyde for 40 min at room temperature, washed with PBS, permeated with PBS containing 0.1% Triton X-100, and stained with 2.5 μg/ml DAPI (Molecular Probes, Eugene, OR, USA). Cell number per blastocyst was counted under epifluorescence microscope (Olympus, Japan).

### Experimental Design

Experiment 1 was designed to evaluate the effect of different concentrations of DMSO and EG in vitrification solutions. The concentrations of DMSO and ethylene glycol (EG) in VS1 and VS2 were described in Table 1. All solutions used during vitrification were prepared with holding medium (HM) consisting of 25 mM Hepes buffered TCM199 containing 20% FBS at 38.5℃. The blastocysts derived from nuclear transfer with or without lipid were vitrified in each medium of different concentrations of DMSO and EG. Five to seven blastocysts were loaded into an open pulled straw (OPS, Minitüb, Germany) and immediately plunged into liquid nitrogen with 25 sec. OPSs were stored for a week in liquid nitrogen tank before warming. Vitrified blastocysts were warmed by immersing the end of the OPS into 1.2 ml WS1 containing 0.3 M sucrose in HM for 5 min at 38.5℃, then transferred blastocysts to WS2 containing 0.15 M sucrose in HM for 5 min at 38.5℃, and washed blastocysts with HM for 5 min, twice, and transferred into PZM-3 medium containing 0.3% BSA for further culture (Fig. 1).

Experiment 2 was designed to evaluate the effect of different concentrations of sucrose in warming solution. The concentrations of sucrose in WS1 and WS2 were described in Table 2. The blastocysts derived from nuclear transfer with or without lipid were placed in first vitrification solution with 10% DMSO and 10% EG for 2 min, then second vitrification solution with 20% DMSO and 20% EG for 25 sec, and loaded into OPS. A straw was warmed into different concentrations of sucrose in WS1 and 2 for 5 min at 38.5℃, respectively, and washed blastocysts with HM for 5 min, twice, and transferred into PZM-3 medium containing 0.3% BSA

**Table 1. Vitrification solution of experimental design 1**

| Treatment | Vitrification solution 1 (VS1)[1] | | Vitrification solution 2 (VS2)[2] | |
|---|---|---|---|---|
| | DMSO (%) | EG (%) | DMSO (%) | EG (%) |
| A | 5 | 5 | 10 | 10 |
| B | 10 | 10 | 20 | 20 |
| C | 15 | 15 | 30 | 30 |

[1] Oocytes were incubated in VS1 for 2 min, and then transferred into VS2.

[2] Oocytes were incubated in VS2 for 25 sec, and transferred into open pulled straws (OPS), immediately.

**Table 2. Warming solution of experimental design 2**

| Treatment | Concentration of sucrose (M) | |
|---|---|---|
| | Warming solution 1 (WS1)[1] | Warming solution 2 (WS2)[2] |
| A | 0.5 | 0.25 |
| B | 0.3 | 0.15 |
| C | 0.1 | 0.05 |

[1] Oocytes exposed to WS1 for 5 min, and then transferred into WS2.

[2] Oocytes were incubated in WS2 for 5 min.

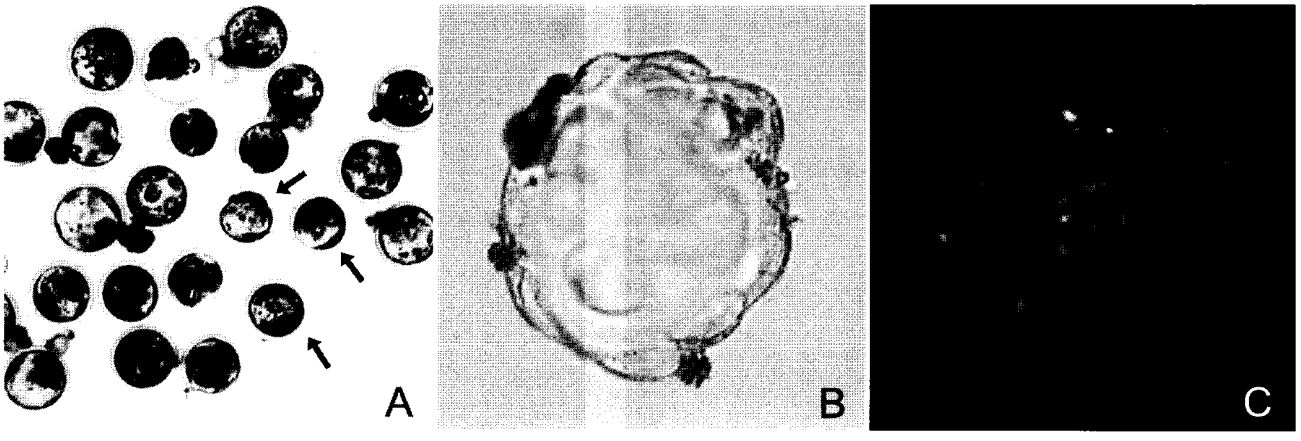


**Fig. 1. Porcine NT blastocysts before and after vitrification.** A: Blastocysts (arrows) derived from NT were used for vitrification. B: An hatched blastocyst after vitrification, rehydration and culture for 24 hr after thawing. C: Nuclei of an expanded blastocyst stained with DAPI.

for further culture.

**Statistical Analysis**

Analyses of variable (ANOVA) were carried out using the SAS package in a completely randomized design. Duncan's multiple range test was used to compare values of individual treatment, when the F-value was significant ($p< 0.05$).

## RESULTS

**Effects of Vitrification Solutions on Porcine NT Blastocysts**

The effect of vitrification solutions on survival of blastocysts derived from NT oocytes with or without lipid removal was summarized in Table 3. There were no significant differences on the number of blastocysts and cell number in blastocysts after thawing and culture among different concentrations of vitrification solutions. The percentage of expanded blastocyst after thawing and culture was significantly higher in the treatment B than in the treatment A and C. In the treatment B, the percentage of expanded blastocyst was significantly higher when cryopreserved after lipid removal. The percentage of degenerated blastocyst in the lipid removal treatments was lower compared to that in the lipid non-removal treatments.

**Effects of Warming Solutions on Porcine NT Blastocysts**

The effect of warming solutions on survival of blastocysts derived from NT oocytes with or without lipid removal was summarized in Table 4. There were no significant differences on the number of blastocysts and cell number in blastocysts after thawing and culture among different sucrose concentrations of warming solutions. The percentage of expanded blastocyst after thawing and culture in the treatment B was higher than that in the treatment A and C. Especially, the highest percentage of expanded blastocyst showed in the treatment B with lipid removal. Also, the rates of degenerated blastocyst in the treatment B with or without lipid removal were significantly lower than those in the treatment A and C without lipid removal.

## DISCUSSION

The ultra-rapid cooling rate provided by the open pulled straw (OPS) vitrification minimized the cryoinjury by rapid passage of the temperature-sensitive zone (Vajta *et al.*, 1998). OPS procedure is currently being studied in several species to solve the problems of cryopreservation of highly sensitive embryos. Berthelot *et al.* (2000) and Cuello *et al.* (2004) have reported that the OPS vitrification system is effective for preserving early or expanded porcine blastocysts produced *in vivo*. Few studies are available on the effects of vitrification of *in vitro* produced porcine embryos, especially somatic cell nuclear transfer embryos (SCNT). Among the several permeating cryoprotectants, dimethyl sulfoxide (DMSO) has been most frequently used in vitrification (Kono *et al.*, 1988; Berthelot *et al.*, 2000).

**Table 3. Effect of vitrification solutions on survival of porcine blastocysts derived from nuclear transfer embryos***

| Treatment of vitrification solution[1] | Lipid removal of oocytes[2] | No. of blastocyst vitrified | Morphology of blastocyst thawed and cultured for 24 hr | | | Cell no. per blastocyst (Mean±SE) |
|---|---|---|---|---|---|---|
| | | | No. of blastocyst (%) | No. of expanded blastocyst (%) | No. of degenerated blastocyst (%) | |
| A | - | 17 | 10(58.8) | 0(0.0)$^{c}$ | 7(41.2)$^{a}$ | 26.1±2.6 |
| | + | 16 | 13(81.3) | 0(0.0)$^{c}$ | 3(18.7)$^{ab}$ | 24.3±1.2 |
| B | - | 17 | 10(58.8) | 2(11.8)$^{b}$ | 5(29.4)$^{ab}$ | 24.3±2.0 |
| | + | 17 | 9(53.0) | 7(41.1)$^{a}$ | 1( 5.9)$^{b}$ | 23.0±1.2 |
| C | - | 17 | 11(64.7) | 0(0.0)$^{c}$ | 6(35.3)$^{ab}$ | 23.1±3.0 |
| | + | 17 | 12(70.6) | 0(0.0)$^{c}$ | 5(29.4)$^{ab}$ | 19.5±0.8 |

[1] A: 5% DMSO and 5% EG in VS1, 10% DMSO and 10% EG in VS2; B: 10% DMSO and 10% EG in VS1, 20% DMSO and 20% EG in VS2; C: 15% DMSO and 15% EG in VS1, 30% DMSO and 30% EG in VS2.

[2] -: lipid non-removal; +: lipid removal.

* Experiments were repeated three times.

[a~c] Means with different letters in the same column differ significantly ($p<0.05$).

**Table 4. Effect of warming solutions on survival of porcine blastocysts derived from nuclear transfer embryos***

| Treatment of warming solution[1] | Lipid removal of oocytes[2] | No. of blastocyst vitrified | Morphology of blastocyst thawed and cultured for 24 hr | | | Cell no. per blastocyst (Mean±SE) |
|---|---|---|---|---|---|---|
| | | | No. of blastocyst (%) | No. of expanded blastocyst (%) | No. of degenerated blastocyst (%) | |
| A | - | 17 | 9(52.9) | 2(11.8)$^{bc}$ | 6(35.3)$^{a}$ | 24.1±1.8 |
| | + | 17 | 12(70.6) | 3(17.6)$^{bc}$ | 2(11.8)$^{bc}$ | 19.3±0.5 |
| B | - | 19 | 14(73.7) | 5(26.3)$^{ab}$ | 0( 0.0)$^{c}$ | 25.9±5.9 |
| | + | 17 | 11(64.7) | 6(35.3)$^{a}$ | 0( 0.0)$^{c}$ | 23.1±1.3 |
| C | - | 20 | 14(70.0) | 0( 0.0)$^{c}$ | 5(30.0)$^{ab}$ | 26.2±3.0 |
| | + | 17 | 12(70.6) | 3(17.6)$^{bc}$ | 2(11.8)$^{bc}$ | 24.9±2.0 |

[1] A: 0.5 M sucrose in WS1 and 0.25 M sucrose in WS2; B: 0.3 M sucrose in WS1 and 0.15 M sucrose in WS2; C: 0.1 M sucrose in WS1 and 0.05 M sucrose in WS2.

[2] -: lipid non-removal; +: lipid removal

* Experiments were repeated three times.

[a~c] Means with different letters in the same column differ significantly ($p<0.05$).

Ethylene glycol (EG) is now widely used for various mammalian species as a low toxicity cryoprotectant (Kasai, 1997).

Li *et al.* (2006) reported that Day 5 and Day 6 blastocysts were placed in equilibration solution (10% DMSO + 10% EG) for 2 min, followed by exposure to vitrification solution (20% DMSO + 20% EG). Also, the embryos developed to the re-expanded blastocyst stage and survived cryopreservation. In this study, the percentages of expanded blastocyst in the treatment vitrified in 10% DMSO + 10% EG and 20% DMSO + 20% EG were significantly higher than those of expanded blastocysts in the treatments vitrified in 5% DMSO + 5% EG and 10% DMSO + 10% EG, and 15% DMSO + 15% EG and 30% DMSO + 30% EG.

Non-permeating sucrose reduces the rate of rehydration during the removal of cryoprotectants. Li *et al.* (2006) reported that embryos were warmed by immersing the end of the OPS straw into 0.3 M sucrose for 5 min at 38.5℃, transferring them to 0.2 M sucrose for 5 min, and then holding medium for 5 min. In this study, the percentages of expanded blastocysts in the treatment warmed in 0.3 M sucrose + 0.15 M sucrose were significantly higher than those of expanded blastocyst in the treatment warmed in 0.5 M sucrose + 0.25 M sucrose and 0.1 M sucrose + 0.05 M sucrose.

Embryos at the perihatching stage including the expanded blastocyst stage were most cryotolerant. The lipid droplets were abundant in 1- to 8- cell stage porcine embryos, but they declined markedly at the perihatching stage (Niimura and Ishida, 1980). Our results showed that use of the OPS method in conjunction with lipid removal provided effective cryopreservation of porcine nuclear transfer embryos. It was found that lipid removal of oocytes significantly improved the viability after cryopreservation. Nagashima *et al.* (1995) reported that high levels of intracellular lipid in porcine oocytes and embryos were associated with their extreme sensitivity to low temperature. In general, the presence of lipid droplets in the cytoplasm is considered an important aspect for embryonic development in terms of energy metabolism (Leese, 1991). The mean cell numbers of the delipated porcine embryos tended to be lower than those for control embryos without lipid removal (Nagashima *et al.*, 1994; Men *et al.*, 2005). However, Nagashima *et al.* (1995) reported that lipid removal of *in vitro* produced porcine embryos did not appear to pose a problem because lipid removal embryos have produced progeny.

In conclusion, we found out that OPS vitrification system was effective for porcine blastocyst produced *in vitro*. The optimal concentrations of cryoprotectants in vitrification solution were 10% DMSO + 10% EG in VS1 and 20% DMSO + 20% EG in VS2. The optimal concentrations of sucrose were 0.3 M sucrose in WS1 and 0.15 M sucrose in WS2. Also, lipid removal of oocytes significantly improved the viability after cryopreservation.

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