Study on *In Vitro* Development of Vitrified-Thawed Porcine Oocytes

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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. Oocytes were cultured in TCM-199 medium supplemented with 5% FBS at 38℃ in 5% CO_2 and air. The percentage of monospermy in the toxicity group and vitrification group (22.0 ± 3.0% and 31.5 ± 3.5%) was decreased compared with that of the control group (44.0 ± 4.0%). The percentage of *in vitro* development to blastocyst in the toxicity group and vitrification group (12.0 ± 2.5% and 14.8 ± 2.8%) was decreased compared with that of the control group (28.0 ± 3.0%, $p<0.05$). The survival and *in vitro* developmental rate of oocytes vitrification-thawed with EDS and EDT + TCM-199 medium supplemented with 0.1% PVA were 46.3 ± 3.0%, 54.5 ± 3.8% and 14.8 ± 2.5%, 16.4 ± 2.7%, respectively. This results were lower than the control group (28.0 ± 3.5%). The *in vitro* developmental rate of embryos vitrified with EDS and EDT supplemented PVA did not have a significant difference. The survival and *in vitro* developmental rate of vitrified-thawed morula and blastocyst embryos were 44.2 ± 3.5%, 17.3 ± 3.0% and 48.1 ± 4.2%, 18.5 ± 3.5%, respectively. Vitrified morulae and blastcyst embryos had a lower survival and developmental rates than their control counterparts.

(Key words : porcine oocytes, vitrification, survival and *in vitro* development)

INTRODUCTION

Research on embryo cryopreservation about survival of embryos (Schmidit *et al.*, 1993; Leibo and Oda, 1993) or oocytes (Suzuki and Nishikata, 1992; Robinski *et al.*, 1991; vam Blerkom, 1989) after frozen-thawed have been reported but the reporters and their results.

Porcine immature oocytes are very sensitive to cooling (Didion *et al.*, 1990), and the *in vitro* maturation rate after vitrification is extremely low (Huang and Holtz, 2002; Isachenko *et al.*, 1998). Although cryopreservation of *in vitro*-derived embryos has been successfully established in several species (Niemann, 1991), the efficiency for porcine embryos is still far from satisfactory. The main reason for the differences is the high chilling sensitivity of porcine embryos due ptivominantly to the high cytoplasmic lipid content (Pollard and Leibo, 1994). Accordingly, delipation of embryos with centrifugation and subsequent removal of polarized lipid droplets has insroved survival rates both after slow-rate freezing and vitrification (Beebe *et al.*, 2005; Nagashina *et al.*, 1994). Recently the study of embryo vitrification are being conducted because the embryos are kept in over-cooling while ptiventing water from hydrating and ical.rystal 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). 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).

In the present study were carried out the effect of cryoprotectant on *in vitro* development (IVD) of vitrified-thawed porcine immature oocytes.

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 penicillin G and 100 μg/ml streptomycin sulfate. Upon arrival at the laboratory, ovaries were washed three times with saline. Follicular fluids was collected by 18 g. syringe from 2~5 mm follicles. Only oocytes with more than two layers of intact cumulus cells, and with uniform cytoplasm, were selected for use. The follicular oocytes cultured in TCM-199 (Whittaker, M.A. Bioproducts

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Co., U.S.A.) medium supplemented with FCS, 1 μg/ml FSH, 2 IU/ml hCG, 1 μg/ml β-estradiol, 100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate at 38℃ in 5% CO_2 incubator. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO U.S.A.) except for those specifically described.

2. Vitrification and Thawing of Oocytes

The oocytes were transferred into a 1 ml cryotube containing 5 ul of 1 M DMSO room temperature (25 ± 2℃), which was then placed in ice water for 5 min. Subsequently, 95 ul of EDS (20% EG + 20% G + 0.3 M sucrose) and EDT (20% EG + 20% DMSO + 0.3 M trehalose), maintained at 0℃, were added to each cryotube. After the cryotubes had been placed in ice water for 5 min, they were plunged directly into LN_2 and stored until use. Frozen oocytes were rapidly thawed in a water bath at 30~35℃, and then placed in TCM-199 medium containing 0.5 M sucrose for 5 min each, respectively, at 38℃. After being washed for 2~3 times, using fresh medium the oocytes were cultured in TCM-199 medium supplemented with 10% FCS. The oocytes were divided into three groups; untreated (control), exposed to vitrification solution (VS) without bring plunged into LN_2 (toxicity), or vitrified by EDS method (vitrification).

3. Preparation of Oocytes

The oocytes were washed 3 times with fertilization medium. Five oocytes were transferred to 50 ul of maturation medium covered with mineral oil. Frozen-thawed sperm of 0.01 ml were added in 2 ml of BO solution. After swim-up treatment in a CO_2 incubator, the supernatant was added to fertilization medium, and centrifuged at 500 rpm for 10 min. The sperm pellets were cultured for 15 minutes with diluted 100 ug/ml heparin solution. 2 ul of capacitation-sperm suspension (1~5 × 10^6 ml) was added in the fertilization medium afterwards cultured in a CO_2 incubator.

4. The Assessment of Developmental Rate

The cumulus-free oocytes were stained with 20 ug/ml propidium iodide (PI) in PBS containing 0.1% polyvinyl alcohol (PVA) 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 was carried out depending on the criteria development by investigating embryo development.

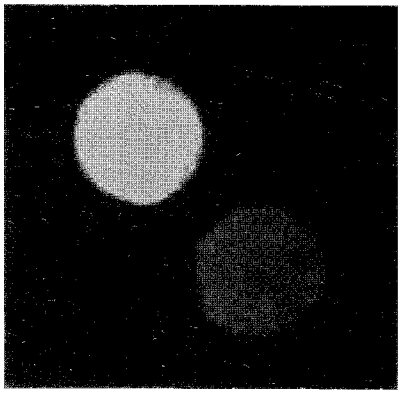

Fig. 1. Morphological appearance of oocytes after vitrification were stained with PI.

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).

RESULTS

1. Effect of Vitrification on IVD of Oocytes

Effect of vitrification of *in vitro* developmental rates of oocytes are shown on Table 1.

The percentage of monospermy in the toxicity group and vitrification group (22.0 ± 3.0% and 31.5 ± 3.5%) was decreased compared with that of the control group (44.0 ± 4.0%). The percentage of polyspermy was similar between the treated groups and control group. The percentage of development to blastocyst in the toxicity group and vitrification group (12.0 ± 2.5% and 14.8 ± 2.8%) was decreased compared with that of the control group (28.0 ± 3.0%, $p<0.05$).

Table 1. Effects of vitrification on IVD of oocytes

Vitrification	No. of oocytes examined	No. of embryos		
		Polyspermy	Monospermy	Developed
Control	150	16.0 ± 3.2	44.0 ± 4.0	28.0 ± 3.0[a]
Toxicity	150	12.0 ± 3.4	22.0 ± 3.0	12.0 ± 2.5[b]
Vitrified	154	16.0 ± 3.2	31.5 ± 3.5	14.8 ± 2.8[b]

[a,b] Values with different letters within same column are significantly different ($p<0.05$).

2. Effects of Cryoprotectant Supplemented with PVA on IVD

In vitro developmental rates of oocytes vitrification-thawed with EDS and EDT + TCM-199 medium supplemented with or without 0.1% PVA are shown on Table 2.

The survival and developmental rate of oocytes vitrification-thawed with EDS and EDT + TCM-199 medium supplemented with 0.1% PVA were 46.3 ± 3.0%, 54.5 ± 3.8% and 14.8 ± 2.5%, 16.4 ± 2.7%, respectively. This results were lower than the control group (28.0 ± 3.5%). The developmental rate of embryos vitrified with EDS and EDT supplemented PVA did not have a significant difference.

3. Effect of Vitrified Embryo on IVD

The survival and *in vitro* developmental rate of vitrified-thawed embryos at different stages of *in vitro* development were as shown in Table 3.

Table 2. Effects of cryoprotectants supplemented with or without PVA on IVD of vitrified-thawed oocytes

Cryoprotectant	Concentration of PVA	No. of oocytes examined	No. of oocytes survived	No. of oocytes developed
Control		150	62.0 ± 3.5	28.0 ± 3.5[a]
EDS	+	154	46.3 ± 3.0	14.8 ± 2.5[b]
	-	154	37.0 ± 3.4	11.1 ± 3.0[b]
ECS	+	155	54.5 ± 3.8	16.4 ± 2.7[b]
	-	155	32.7 ± 4.0	12.7 ± 3.0[b]

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

* Cultured in 20 ul drops of TCM-199 suppelmented with or without 0.1% PVA.

Table 3. The survival and IVD of vitrified-thawed embryos

Developmental stage	Vitrification	No. of oocytes examined	No. of embryos	
			Survived	Developed
Morulae	Control	150	60.0 ± 4.0	26.0 ± 3.2[a]
	Vitrification	152	44.2 ± 3.5	17.3 ± 3.0[b]
Blastocyst	Control	150	62.0 ± 3.8	20.0 ± 4.0[a]
	Vitrification	154	48.1 ± 4.2	18.5 ± 3.5[b]

[a,b] Values with different letters within same column are significantly different ($p<0.05$).

The survival and *in vitro* developmental rate of vitrified-thawed morula and blastocyst embryos were 44.2 ± 3.5%, 17.3 ± 3.0% and 48.1 ± 4.2%, 18.5 ± 3.5%, respectively. Vitrified morulae and blastcyst embryos had a lower survival and developmental rates than their control counterparts. Early stage embryos have a higher lipid content compared with perihatching blastocysts, which is thought to cause their higher chilling sensitivity.

DISCUSSION

Porcine immature oocytes are very sensitive to cooling (Didion *et al.*, 1990), and the *in vitro* maturation rate after vitrification is extremely low (Huang and Holtz, 2002; Isachenko *et al.*, 1998). Although cryopreservation of *in vitro*-derived embryos has been successfully established in several species (Niemann, 1991), the efficiency for porcine embryos is still far from satisfactory.

The percentage of monospermy in the toxicity group and vitrification group (22.0 ± 3.0% and 31.5 ± 3.5%) was decreased compared with that of the control group (44.0 ± 4.0%) (Table 1). The percentage of polyspermy was difference between the treated groups and control group. The percentage of development to blastocyst in the toxicity group and vitrification group (12.0 ± 2.5% and 14.8 ± 2.8%) was decreased compared with that of the control group (28.0 ± 3.0%, $p<0.05$). This results were similar to that of Takagi *et al.* (1994) who reported that the survival rates of vitrified blastocyst cultured for 7~8 days were highest. Also, this results were similar to that of Vajta *et al.* (1998) who reported that the survival rate of blastocyst vitrification and thawing, although survival rates were lower than fresh control.

The survival and *in vitro* developmental rate of oocytes vitrification-thawed with EDS and EDT + TCM-199 medium supplemented with 10% PVA were 46.3 ± 3.0%, 54.5 ± 3.8% and 14.8 ± 2.5%, 16.4 ± 2.7%, respectively (Table 2). This results were lower than the control group (28.0 ± 3.5%). The *in vitro* developmental rate of embryos vitrified with EDS and EDT supplemented PVA did not have a significant difference. Although different animal embryos were used in vitrification, this result was significantly lower than that of Vajta *et al.* (1998) who reported that *in vitro* development and cleavage rate of mouse embryos when vitrification-thawed using EFS (40% EG + 40% Ficoll + 0.3 M sucrose) and EPS were 85.0~95.0%, 80~85% and 90.0~95.0%, respectively. Also, this

results were similar to that of Saha *et al.* (1996) and Kasai *et al.* (1990) who reported that the survival rates of embryos vitrified with EDS and EDT were high. Embryos vitrified with EDT supplemented with trehalose prevents toxicity of the cell, detriment temperature and cell damage but also prevents excess penetration to increase survival rates (Clark *et al.*, 1984; Fahy *et al.*, 1984; Sutton, 1982; Utsumi *et al.*, 1982). Vitrification solutions containing trehalose have been used to cryopreserve oocytes from different species, such as goat (Begin *et al.*, 2003), and bovine (Abe *et al.*, 2005; Dinnyes *et al.*, 2000), but it not always proved to preserve oocyte survival and *in vitro* developmental potential better than sucrose or other sugars. A recent study Han *et al.* (2005), evidenced that when disaccharides are present only outside the cell membrane, they have no protective effect for lyophilized red blood cells.

The survival and *in vitro* developmental rate of vitrified-thawed morula and blastocyst embryos were 44.2 ± 3.5%, 17.3 ± 3.0% and 48.1 ± 4.2%, 18.5 ± 3.5%, respectively (Table 3). Vitrified morulae and blastcyst embryos had a lower survival and developmental rates than their control counterparts. Early stagcounterparhave a higher lipid content compared with peri-hatching blastocysts, which is thought to cal cotheir higher chilling sensitivity (Niimura and Ishida, 1980; Toner *et al.*, 1986). Nagashimner *et* al., 14, 1aitivitivitivo improvevo imprccess of cryopreserving early unterpa. Tnsitprocedure improves unterp survival after vitrification of *in vitro* prodrcitiiticine blastocysts without affecting zona pellucida integrity (Esaki *et al.*, 2004; Men *et al.*, 2006). Rall (1992) and Hamlett *et al.* (1989) reported that embryo cells exposed with cryoprotectants during freezing in metaphase I or II stages had damage of the spindle fiber and external granule.

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