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Developmental Rate of Vitrified Porcine Oocytes and Its Application to NT Embryos Constructed by Microinjection of Fibroblast Cells into Vitrified Oocytes

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돼지 동결 난포란과 이를 이용한 핵이식 배의 체외발생에 관한 연구

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SUMMARY

본 연구는 돼지 난포란의 동결보존 후 생존성과 난자의 활성화 처리에 따른 체외발생율과 이를 이용한 핵 이식배의 체외발생율을 조사하였다. 활성화 처리된 배는 5% FBS가 첨가된 NCSU 23 배양액으로 38.5℃, 5% CO_2와 95% air의 조건으로 배양하였다.

1. 난포란을 EDS와 5% PVP로 동결 후 10% FBS가 첨가된 NCSU 23 배양액으로 0~10시간 배양했을 때 체외발생율은 36.0%로서 대조군인 비동결 난포란의 체외발생율 46.0%에 비해 낮았다.
2. Ethanol과 cyclojexamide로 처리 후 42 및 46시간 배양한 배의 분할율은 각각 33.3%, 36.0% 및 27.7%, 30.0%로서 대조군의 8.8%, 11.4%에 비해 높게 나타났다.
3. 동결 및 비동결 난포란을 이용한 핵이식 배의 융합율과 발생율 간에는 유의한 차이가 없었다.
4. Ethanol과 cyclojexamide로 활성화 처리한 난자를 이용하여 재구축한 핵 이식배의 발생율은 2.8%, 5.3% 및 1.5%, 2.9%로서 대조군의 0.0%, 0.0%에 비해 높은 발생율을 나타냈다.

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

INTRODUCTION

Research on embryo cryopreservation about survival of oocytes (Robinski *et al.*, 1991; van Blerkom, 1989) or embryos (Leibo 1993; Cuello *et al.*, 2004) after frozen-thawed have been reported but there was much difference between the reporters and their results.

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(Kasai *et al.*, 1990; Vajta *et al.*, 1998; Cuello *et al.*, 2004) reported that oocyte cells exposed with cryoprotectants during freezing in metaphase I or II

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stages had damage of the spindle fiber and external granule. Mazur(1970) reported that during freezing, the main reason that the cells died was because the in the cell had ice crystallization and thawing influence and because of this have reported that reasonable equilibrium time is needed. Rall and Fay (1985) reported that freezing the early 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). Successful vitrification requires high concentration of permeable cryoprotectants and a very rapid cooling rate. Accelerating the cooling rate could decrease the cryoprotectants concentration required for vitrification(Vajta, G., 1999). The simplest way to accelerate the rates of cooling and warming is to reduce VS volume and establish direct contact between the VS (containing the oocytes) and the cooling/warming solution(Vajta, G., 1999). These treatments are commonly associated with protein synthesis inhibitors, that prevent cyclin synthesis, such as cycloheximide, and phosphorylation inhibitors, which prevent MPF activation, such as 6-dimethylaminopurine(Susko-Parrish *et al.*, 1994; Loi *et al.*, 1998). Among the deficiencies in the process of artificial activation is the dependence on oocyte maturation time (Barnes *et al.*, 1993; Presicce and Yang, 1994; Soloy *et al.*, 1997; Bordignon and Smith, 1998). Activation is more easily achieved in aged oocytes when compared with those more recently activated, due to the spontaneous reduction on MPF activity with aging of oocytes(Kikuchi *et al.*, 1995: Wu *et al.*, 1997). Oocyte activation allows cell cycle phase synchronization between the cytoplasm of the oocyte and the transferred nucleus, promoting nuclear reprogramming and maintenance of correct ploidy. In bovine, strontium has been shown to induce multiple intracellular calcium spikes in a fashion similar to that of fertilization(Bos-Mikich *et al.*, 1995) and has been efficiently used to induce activation of oocytes (Cuthbertson *et al.*, 1981; Bos-Mikich *et al.*, 1995) and embryos reconstructed by nuclear transfer(Quan *et al.*, 2005; Wakayama *et al.*, 1998). To date, no information has been made available on the use of cryopreserved porcine oocytes as recipient cytoplasts for somatic cell NT.

Therefore the present 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 NT, and observed that porcine oocytes are activated by ethanol and strontium as evaluated by pronuclear formation, and parthenogenetic development.

MATERIALS AND METHODS

1. Preparation of Oocytes

Oocytes collected form 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 and 10 ng/mL EGF 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(North Carolina State University 23) supplemented with 10% FCS and 50 μg/mL of gentamicin sulfate for 15 min on a hot plate 38℃. They were then immersed in a 40% ethylene glycol, 16.5% DMSO, 0.5 M sucrose(EDS), 5% PVP in NCSU 23 supplemented with 10% FCS and 50 ug/mL of gentamicin sulfate. Vitrification oocytes are cultured in VS_1 solution for 1 min., afterwards transferred to a 20 μL drop VS_2 solution, and then

quickly added to the EDS solution to expose for 1 minute. The oocytes were sealed in a 1.0mm OPP straw(Vajta *et al.*, 1998a) and placed in a LN_2 container. Frozen Oocytes were rapidly thawed in a water bath at 30~35℃, and then placed in 0.5 M sucrose, 0.5 M galactose and 0.5 M trehalose for 5 min each. After being washed for 2~3 times, using fresh medium the oocytes were cultured in a 10% FAC+TCM-199 medium.

After exposure for 30~35 s, 3 μL of VS containing in liquid nitrogen using a manipulating micropipette. The vitrified microdrops were transferred to a pre-cooled 2 mL cryogenic vial(Asahi Tec. Glass Co., Japan) and stored in liquid nitrogen for 10 days. The vitrified oocytes were poured back on a pre-cooled aluminium foil floating in LN_2 and transferred 2 mL of dilution medium consisting of 0.3 M sucrose in NCSU 23 supplemented with 10% FCS and 50 μg/mL of gentamicin sulfate for 15 min on a hot plate at 38℃. Survival of oocytes was morphologically evaluatedafter 2 h of culture in an embryos culture medium: NCSU-23 culture medium supplemented with 5% FBS, 10 IU/mL hCG, 10 IU/mL eCG, and 10 ng/mL EGF at 38℃ under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

3. Enucleation of Oocytes

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 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.

4. Preparation of Nuclear Donor Cells

Matured porcine oocytes and ear-skin derived fibroblast were used as nuclear donor cells for NT. To establish donor nuclei cell lines, limb and ear-skin tissues were collected from a 40-day porcine fetus. Sliced tissue was washed in PBS, vortexed for 3 min in D-PBS medium (Gibco, USA) supplemented with 0.25% trypsin/EDTA (Gibco, U.S.A.), and centrifuged with NCSU-23 medium (Sigma, U.S.A.) supplemented with 5% FBS (Gibco, U.S.A.). After removal of the trypsin and EDTA, separated cells in NCSU-23 medium supplemented with 5% FBS were cultured at 38℃ in a CO_2 incubator (5% CO_2, or 5% O_2 and 90% air). When a cultured cell successfully formed a monolayer on a dish, serum starvation culture in NCSU-23 medium supplemented with 5% FBS was carried out for 3 days. Induced Go or G1 stage cells cultured for 15~20 days were used as donors of nuclei in subsequent experiments.

5. Nuclear Transfer and Culture

Cultured donor oocytes were treated with 0.25% trypsin-EDTA, and separated single cells were suspended in NCSU 23 drops supplemented with 5% FBS. Recipient oocytes were grasped with a holding pipette of a micromanipulator. Fibroblast cells were pre-synchronized in medium supplemented with 0.2% serum for 48 h or 0.5% and 1% serum for 7 days. Pre-synchronization in early S-phase before incubation in medium containing 0.1 μg/m Hoechst 33342 an increase from 8~15 h culture an increased percentage of porcine fibroblast cells in G2/M at the end of the synchronization period. Donor oocytes were loaded into a 20~30 μm pipette and injected singly into the zona pellucida and vitelline space of the recipient cells. Electro cell fusion(LF-100, Life Tec. Co. Japan) between rwo wire electrodes overlaid with 0.3 M mannitol (Sigma, USA) solution containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$. These cells were then fused using direct current 1.90 kv/cm for 30 sec, then alternating current for 5 v/mm 5 sec, and with count

current 1.50 kv/cm for 30 sec. Nuclear transferred embryos were treated with 5 g/mL ionomycion, activated with 1.5 mM 6-DMAP for 4 hr, cultured in NCSU-23 or TL-Heaps medium (both containing 3 mg/mL BSA) in a CO_2 incubator. CHX-treated couplets were cultured in the embryos culture medium for 7 days to determine *in vitro* development to the blastocyst stage. All of the blastocysts obtained at the end of culture were prepared for cell counting by an air-dry method(Takahashi and First, 1992).

6. Activation Treatments of Oocytes

Before activation, oocytes were washed three times in Hepes buffered NCSU 23 +10% FCS(Sigma, USA) and then activated by ethanol, incubation for 5 min in and cyclohexamide, cultured for 5 h in calcium-free NCSU 23 medium. After activation treatments, the oocytes were transferred to development culture drops of 100 μL NCSU 23 with 10 mg/mL BSA and 75 μg/mL kanamycin, under mineral oil, and cultured at 38°C under an atmosphere of 5% CO_2 in air up to 40~48 h until assessment procedures to determine activation and cleavage rates.

7. 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 μg/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.

8. 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).

RESULTS

1. Developmental Rate of Vitrified Oocytes

In vitro developmental rates of porcine oocytes vitrified with EDS, 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 were 50.0%, 22.0%, respectively. This results were lower than the control group(74.0%).

2. 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 2. 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. However, the mean cell number in the blastocysts derived from vitrified oocytes was lower than that of the non-vitrified control($p<0.05$). After thawing, 89.6± 8.3% of vitrified oocytes were recovered, and 88.1± 2.1% of these were judged as viable. The most common morphological damage to oocytes was the

Table 1. *In vitro* developmental rate of vitrified porcine oocytes with EDS solution

Treatment of oocytes	No. of oocytes vitrified	No. of oocytes thawed	No. of oocytes developed (%)
Control	50	37(74.0)[a]	23(46.0)
Vitrified	50	32(64.0)	18(36.0)

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

** EDS(40% EG+20% DMSO +0.4M sucrose) 5% PVP, NCSU 23 supplemented with 10% FBS solution.

Table 2. Developmental rate of 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	No. of BL cell
Vitrified	80	61.3±2.4	40.0±2.0	17.5±2.7	1052±6.2
Non-vitrified	72	65.3±1.8	54.2±1.7	23.6±2.4	1635±6.0

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

rupture of the zona pellucida with a loss of cytoplasm or degenerated cytoplasm. No significant differences were observed in the rate of enucleation between vitrified(61.3±2.4%) and non-vitrified oocytes(65.3±1.8%).

3. Developmental Rate of NT Oocytes Activated with Ethanol and Cyclohexamide

Cleavage and developmental rates of NT embryos constructed with oocytes activated with ethanol and cyclohexamide are shown in Table 3.

Cleavage rates after of NT embryos constructed with oocytes activated with ethanol and cyclohexamide were superior ($p>0.05$) for ethanol (33.3%), cyclohexamide (27.7%) treatments and the control (21.3%). After maturation for 42 and 46 h, cleavage rates for ethanol (33.3 and 36.0%) and cyclohexamide (27.7 and 30.0%) were to the control (8.8 and 11.4%). Oocyte age influenced cleavage in all groups. For all activation times, there were no significant differences ($p>0.05$) between treatments and the control regarding irregular cleavage. Developmental rates to blastocysts of NT embryos constructed with oocytes activated with ethanol and cyclohexamide were superior for ethanol(2.8 and 5.3%, respectively), cyclohexamide(1.5 and 2.9%, respectively) treatments than that of control(0.0 and 0.0%, respectively).

Table 3. Cleavage and developmental rare of NT embryos constructed with oocytes activated with ethanol and cyclohexamide

Activation	Activation time	No. of oocytes examined	No. of embryos cleavage	No. of embryos developed BL(%)
Control	42	80	7(8.8)	0(0.0)
	46	70	8(11.4)	0(0.0)
Ethanol	42	72	24(33.3)	2(2.8)
	46	75	27(36.0)	4(5.3)
Cyclohex amide	42	65	18(27.7)	1(1.5)
	46	70	21(30.0)	2(2.9)

DISCUSSION

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 (Kasai *et al.*, 1990; Vaita *et al.*, 1998; Cuello *et al.*, 2004). When the developmental rates of the oocytes after being culture for 0~10 hours vitrified with EDS were 50.0%, 22.0%, respectively. This results were lower than the control group (74.0%). 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. The fertilization and development rate of embryos when vitrified using EFS solution

(35.0%, 25.0%) was little higher than that of EPS solution(30.0%, 22.5%), and this result was similar that of Vajta *et al.*(1998) and Kasai *et al.*(1990).

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. 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. Rapid cooling and warming reduce the exposure time to the critical temperature and allow no time for spindle depolymerization (Vajta *et al.*, 1998; Martino, 1996). Alteration of spindle structures results in a scattering of chromosomes(Saunders and Parks, 1999), and some chromatin materials could not be removed during enucleation.

The present intraspecies bovine NT embryos constructed with vitrified bovine oocytes had similar development to those constructed with non-vitrified bovine oocytes(Cuthbertson *et al.*, 1981). This suggests the presence of alterations in the cytoplasmic components of the vitrified oocytes and the necessity for more experiments to determine the full-term development of intraspecies NT embryos constructed with vitrified oocytes.

Cleavage rates after of NT embryos constructed with oocytes activated with ethanol and cyclohexamide were superior ($p>0.05$) for ethanol (33.3%), cyclohexamide (27.7%) treatments and the control (21.3%). Results of the present study indicate that there is no significant difference between ethanol and cyclohexamide treatments regarding pronuclear formation and cleavage rates (Table 3). The number, frequency, amplitude and duration of Ca^{2+} pulses influence the efficiency of activation (Vitullo and Ozil, 1992; Loi *et al.*, 1998) and regulate later developmental events, such as blastocyst development (Ducibella *et al.*, 2002; Grupen *et al.*, 2002; Ikumi *et al.*, 2003). Ethanol promotes a single intracellular Ca^{2+} increase of greater and longer amplitude than the initial increase observed at fertilization (Vitullo and Ozil, 1992; Nakada and Mizuno, 1998; Grupen *et al.*, 2002) and originates both from extracellular entry of Ca^{2+} and mobilization of intracellular stores (Loi *et al.*, 1998).

In the present study, parthenogenetic development to the blastocyst stage was similar for ethanol, cyclohexamide and control group. Ethanol promotes a single intracellular Ca^{2+} increase of greater amplitude than the pulses promoted by fertilization and by strontium treatment (Kline and Kline, 1992; Vitullo and Ozil, 1992; Nakada and Mizuno, 1998; Jellerette *et al.*, 2000; Grupen *et al.*, 2002). Ethanol treatment is influenced by oocyte age. Therefore, the more desirable results obtained for ethanol treatment could be due to aging of the oocytes, which promotes a spontaneous decrease in MPF activity (Kikuchi *et al.*, 1995; Wu *et al.*, 1997).

CONCLUSION

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 NT, and observed that porcine oocytes are activated by ethanol and strontium as 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. Activated embryos were cultured in NCSU-23 medium supplemented with 5% FBS, at 38.5℃ for 6 to 8 days in 5% CO_2 and air.

1. When the *in vitro* developmental rates of the oocytes after being culture for 0~10 hours vitrified with EDS, 5% PVP, NCSU 23 supplemented with 10% FBS were 50.0%, 22.0 %, respectively. This results were lower than the control group(74.0%).
2. Cleavage rates of embryos activated with ethanol and cyclohexamide were superior for ethanol (33.3% and 36.0%), cyclohexamide(27.7 % and 30.0 %) treatments than that of control (8.8% and 11.4%).
3. The fusion and developmental rates 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.
4. Developmental rates to blastocysts of NT embryos constructed with oocytes activated with ethanol and cyclohexamide were superior for ethanol(2.8% and 5.3%), cyclohexamide(1.5 %, 2.9%) treatments than that of control(0.0% and 0.0%).

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