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In Vitro Development of Porcine *In Vitro* Matured Oocytes Vitrified after Removal of Cytoplasmic Lipid Droplets

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Abstract: The objective of the present study was to investigate the *in vitro* developmental competence of porcine *in vitro* matured (IVM) oocytes vitrified after removal of cytoplasmic lipid droplets (delipation). After vitrification and warming, the delipated porcine IVM oocytes were inseminated and subsequently cultured *in vitro*. The rate of development to the blastocyst stage of delipated, vitrified oocytes (5.9%) was significantly lower than that of control oocytes (untreated oocytes) (26.2%). We also examined the influence of delipation of porcine IVM oocytes on development to the blastocyst stage following *in vitro* fertilization (IVF). Delipated porcine IVM oocytes (not vitrified) were inseminated and subsequently cultured *in vitro*. The rates of development to the blastocyst stage were similar for delipated and undelipated oocytes (21.1% and 26.2%, respectively). The results of the present study showed that delipated, vitrified porcine IVM oocytes can develop to the blastocyst stage following IVF, though blastocyst formation rate was low, and that delipation of porcine IVM oocytes did not negatively affect their development to blastocyst stage.

Key words: Delipation, Pig, Oocyte, Vitrification, Embryo development

Introduction

Some success in cryopreservation of porcine *in vitro* matured (IVM) oocytes at the metaphaseII (MII) stage has been achieved with vitrification methods [1–4]; however, the developmental competence of porcine IVM oocytes after vitrification is greatly impaired. The presence of a large number of intracytoplasmic lipid droplets in the oocytes has been considered an obstacle to cryopreservation of porcine oocytes, as lipid reduces the cyrotolerance of the oocytes and causes irreversible damage to the membrane structure at low temperatures [5]. Nagashima *et al*. [6, 7] showed that delipation, the removal of cytoplasmic lipid droplets from embryos, remarkably improves embryo viability following cryopreservation, and it has subsequently been shown that the cyrotolerance of porcine germinal vesicle-stage oocytes [8, 9] and early-stage embryos [5, 10, 11] can be dramatically increased by delipation. On the other hand, to our knowledge, there have been very few studies of the developmental competence of delipated porcine IVM oocytes at the MIIstage after cryopreservation.

Our vitrification system is a simple method which vitrifies embryos on a membrane filter which absorbs extracellular vitrification solution [12]. We have reported that the fertility of delipated porcine IVM oocytes cryopreserved by our vitrification system was not different from that of undelipated fresh oocytes [13]. However, no data are available regarding developmental competence after IVF of delipated, vitrified porcine IVM oocytes cryopreserved using our vitrification system. The objective of this study was to investigate the *in vitro* developmental competence of delipated, vitrified porcine IVM oocytes following IVF. Furthermore, we examined the influence of delipation of porcine IVM oocytes on development to the blastocyst stage following IVF.

Materials and Methods

In vitro maturation (IVM) of oocytes

Ovaries obtained at a local abattoir were wrapped in a paper towel soaked in 0.9% saline and transported to the laboratory while kept at a temperature of approx. 30 °C. Cumulus-oocyte complexes (COCs) were aspirated from 3–6-mm follicles of ovaries into a 5-ml disposable sy-

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ringe with a 21-gauge needle and were placed in plastic Petri dishes. COCs having at least 3 layers of compact cumulus cells were selected and cultured in maturation medium. The basic maturation medium was modified TYH medium [14], hereafter referred to as TYH-M, which consisted of 115.0 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2 \cdot 2H_2O$, 1.19 mM $MgSO_4 \cdot 7H_2O$, 1.19 mM KH_2PO_4, 25.07 mM $NaHCO_3$, 5.56 mM glucose, 0.25 mM sodium pyruvate, 10.0 mM HEPES (pH 7.4), 65 µg/ml dibekacin sulfate (Meiji-Seika, Tokyo, Japan), 1 mg/ml polyvinylpyrrolidone (PVP, *Mr.* 40,000, Sigma-Aldrich, St. Louis, MO, USA) and $2 \times 10^{-4\%}$ (w/v) phenol red [13]. The maturation medium was TYH-M supplemented with 7.0 mM taurine, 5.0 mM hypotaurine, 0.6 mM L-cysteine, 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical, Tokyo, Japan), 10 IU/ml hCG (Gonatropin; ASKA), 1 mg/ml fetuin (Sigma-Aldrich), 10 ng/ml epidermal growth factor (EGF) and 1.0% 2nd fraction of ultra-centrifuged porcine follicular fluid (pFF). The 2nd fraction of pFF was prepared by ultra-centrifugation of pFF at 220,000 x *g* for 48 h at 4 °C [15]. COCs were cultured for 22 h in 2 ml of maturation medium covered with mineral oil (Sigma-Aldrich) under a gas phase of 5% CO_2, 5% O_2 and 90% N_2 with high humidity of 39 °C. The COCs were subsequently cultured in the maturation medium without fetuin and hormones for an additional 22 h under the same gas conditions. Thirty to forty COCs were cultured in each well.

Removal of cytoplasmic lipid droplet from oocytes

After 44 h of IVM, COCs were treated with 10 units/ml hyaluronidase dissolved in the basic maturation medium and then denuded of cumulus cells by gentle pipetting. Oocytes at the metaphaseII (MII) stage were selected under a stereomicroscope according to the presence of the first polar body. Extrusion of the lipid droplets from the cytoplasm was carried out according to Hara *et al.* [9] with some modification [13]. In brief, the denuded oocytes were centrifuged (10,000 x *g*, 10 min) at 39 °C in modified PB1 medium [16] supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone Laboratories Inc., Logan, Utah, USA), 0.27 M glucose and 7.5 µg/ml cytochalasin B (CB, Sigma-Aldrich). The modified PB1 medium, designated mPB1, contained 1 mg/ml PVP instead of bovine serum albumin (BSA). For lipid removal, the centrifuged oocytes were immediately manipulated in the same medium. The lipid mass in the oocyte was removed by micromanipulation using a beveled suction pipette (diameter, 7.5 µm) attached to a micromanipulator (MO-108; Narishige, Tokyo, Japan) under an inverted microscope [13]. After lipid removal, the oocytes were washed several times and maintained in mPB1 supplemented with 7.5 µg/ml CB for 10 min.

Vitrification

Delipated oocytes were cryopreserved by the vitrification procedure of Momozawa and Fukuda [12] with some modification [13]. The basic medium used for vitrification was mPB1 supplemented with 14.8 mM L-proline, 1 mg/ml fetuin and 0.2 M trehalose. The vitrification solution was the basic medium with 30% (v/v) ethylene glycol (EG), 0.5% (v/v) glycerol (G) and 0.5 M sucrose. The delipated oocytes were exposed for 2.5 min to 200-µl droplets of the basic medium (39 °C). Subsequently, the delipated oocytes were equilibrated with equilibration solutions (solutions A, B and C) at 39 °C in a stepwise manner. The delipated oocytes were exposed first for 35 sec to 500-µl droplets of solution A, which was composed of 7% (v/v) EG and 0.5% (v/v) G in mPB1, then for 30 sec to 500-µl droplets of solution B, which was composed of 14% (v/v) EG and 0.5% (v/v) G in mPB1, and finally for 30 sec to 500-µl droplets of solution C, which was composed of 21% (v/v) EG and 0.5% (v/v) G in PB1. Immediately after equilibration, the delipated oocytes were exposed for 25 sec to 500-µl droplets of the vitrification solution, then loaded into a glass capillary with about 5 µl of vitrification solution and placed on a nitrocellulose membrane filter (13 mm in diameter, 8.0 µl in pore size). Subsequently, the vitrification solution covering the oocytes was absorbed by the membrane filter, and the membrane filter with the oocytes was plunged into liquid nitrogen (LN_2) and placed in a membrane filter container in LN_2. The vitrified oocytes were stored in LN_2 for 7–174 days. The vitrified oocytes were ultra-rapidly thawed, and the cryoprotectants were removed in a stepwise manner at 39 °C. In brief, the membrane filter with the vitrified oocytes was transferred from LN_2 into 2 ml of mPB1 containing 0.5 M sucrose. The recovered oocytes were consecutively transferred to 500-µl droplets of mPB1 supplemented with 0.3 M or 0.15 M sucrose for 2 min. Subsequently, the delipated oocytes were cultured for 2 h in fresh maturation medium. The delipated, vitrified oocytes, delipated oocytes without vitrification and control oocytes were subjected to IVF and IVC. Control oocytes were neither delipated nor vitrified.

IVF and in vitro culture (IVC)

IVF was carried out according to Momozawa *et al.* [13]. Briefly, ejaculated spermatozoa from a Landrace boar were preserved for 18–20 h at 15 °C. After preservation, suspensions of spermatozoa were washed twice by centrifugation at 900 x *g* for 5 min in PVP-free fertilization medium supplemented with 4 mM caffeine (sodium benzoate, Sigma-Aldrich) and 0.5 mM citrate (sodium citrate-trisodium). The fertilization medium was 10 mM

Table 1. *In vitro* development of delipated, vitrified porcine oocytes following IVF

Treatment		No of oocytes examined	No. (%) of oocytes survived[a]	No. (%) of oocytes developed to[b]			
Delipation	Vitrification			≥2 cell (48 h)[c]	≥8 cell (96 h)[c]	Morula (96–120 h)[c]	Blastocyst (120–192 h)[c]
–	–	130	130 (100)[d]	89 (68.5)[d]	67 (51.5)[d]	58 (44.6)[d]	34 (26.2)[f]
+	+	61	34 (55.7)[e]	12 (35.3)[e]	7 (20.6)[e]	5 (14.7)[e]	2 (5.9)[g]
+	–	57	57 (100)[d]	37 (64.9)[d]	29 (50.9)[d]	25 (43.9)[d]	12 (21.1)[fg]

[a]Survival of the vitrified-warmed oocytes was judged by their morphological appearance at 8 h after insemination. [b]The percentage of oocytes developing to each stage is shown as the number of surviving oocytes. [c]Hours after insemination. [d-g]Values in the same column with different superscripts are significantly different ($P < 0.05$ for f and g, and $P < 0.01$ for d and e). Experiments were performed at least five times.

HEPES-buffered Whittingham's modified Tyrode's solution (WTS) [17] (sodium lactate-free) supplemented with 1 mg/ml PVP instead of BSA. The washed spermatozoa were resuspended in 1 ml of PVP-free fertilization medium supplemented with 4 mM caffeine and 0.5 mM citrate. The sperm suspension was diluted 1:1 with the fertilization medium supplemented with 1.0 mg/ml penicillamine (Sigma-Aldrich), 0.6 mM methyl-*β*-cyclodextrin (Sigma-Aldrich), 0.5 mM citrate, 3.0 units/ml heparin (heparin sodium salt) and 2 mg/ml PVP. The spermatozoa were preincubated in 1.0-ml droplets covered with mineral oil under a gas phase of 100% N_2 with high humidity for 2.5 h, and then preincubated under a gas phase of 5% CO_2 for 1 h at 39 °C. After 3.5-h preincubation, a small amount of sperm suspension was introduced into 50-µl droplets of fertilization medium supplemented with 2 mM caffeine and 5 units/ml hyaluronidase to obtain a final concentration of 300 sperm/µl. Subsequently, oocytes were introduced to the droplets of sperm suspension (about 10 oocytes/drop). Spermatozoa and oocytes were coincubated for 8 h in a CO_2 incubator (5% CO_2, 95% humidified air, 39 °C). At 8 h after insemination, the survival of the oocytes was evaluated by morphological examination under a stereomicroscope. Oocytes with a normal spherical shape and a visible perivitelline space were considered to be live oocytes and were selected for further investigation. Surviving oocytes were cultured for 192 h in 50 µl of PZM-5 [18] covered with mineral oil under a gas phase of 5% CO_2, 5% O_2 and 90% N_2 with high humidity at 39 °C. At 48 h after insemination, cleaved embryos were transferred to a fresh culture medium. Cleavage and development to the 8-cell or more advanced stages, to the morula stage and to the blastocyst stage were observed at 48, 96, 96–120 and 120–192 h after insemination, respectively, under an inverted microscope.

Statistical analysis

Percentage data were subjected to an arcsine transformation and analyzed by one-way ANOVA. Treatment differences were determined using Tukey's multiple comparison test.

Results

The results of this study are presented in Table 1. The survival rate of delipated, vitrified oocytes was significantly lower than that of control oocytes (55.7% vs. 100%, $P < 0.01$). When surviving oocytes were cultured *in vitro*, the delipated, vitrified oocytes showed significantly lower rates of cleavage (35.3%) and development to the 8-cell or more advanced stages (20.6%), morula stage (14.7%) and blastocyst stage (5.9%) than those of the control oocytes. On the other hand, delipation treatment did not have a negative effect on the developmental competence. There were no differences between delipated and control oocytes in the rates of cleavage and development to the 8-cell or more advanced stages, morula stage and blastocyst stage.

Discussion

To our knowledge, there have been very few reports on the developmental competence of delipated porcine IVM oocytes at the MIIstage after cryopreservation. In this study, we investigated the *in vitro* developmental competence of delipated, vitrified porcine IVM oocytes. As shown in Table 1, delipated, vitrified porcine IVM oocytes could develop to the blastocyst stage following IVF; however, the survivability and developmental competence of delipated, vitrified oocytes were significantly lower than those of the control oocytes (untreated oocytes). The survival rate of delipated, vitrified oocytes at 8 h after insemination was lower than expected. One

of the characteristics of the vitrification system used in this study is that the vitrification solution did not contain bovine serum. Cryopreservation solutions are normally supplemented with fetal calf serum or bovine serum albumin as a source of protein, which stabilizes the plasma membrane when added at a high concentration [19] and reduces the amount of visible ice in the vitrification medium [20]. In this study, it is thought that the plasma membrane of oocytes became unstable due to the invasive nature of delipation treatment. Thus, bovine serum may be useful as a supplement for vitrification solutions when delipated porcine IVM oocytes are cryopreserved using our vitrification system.

Moreover, the results of this study indicate that even when the lipid droplets were removed from oocytes, the developmental competence of cryopreserved porcine IVM oocytes did not improve. It has been reported that the cyrotolerance of porcine germinal vesicle (GV)-stage oocytes [8, 9] and early-stage embryos [5, 10] can be dramatically increased by delipation. On the other hand, in the cryopreservation of porcine IVM oocytes, Ogawa *et al*. [21] demonstrated that after a combination of cytoplasmic lipid removal and treatment with a microtubule stabilizer, paclitaxel, vitrified porcine IVM oocytes could efficiently develop to the blastocyst stage. However, in their study, the blastocyst formation rate of the delipation-and-paclitaxel group was significantly lower than that of the intact control oocytes. Therefore, for porcine, it appears difficult to achieve a level of developmental competence for vitrified IVM oocytes that is comparable to that of fresh IVM oocytes. Moreover, as shown in Table 1, the cleavage rate of the delipation-and-vitrification group was significantly lower than that of the controls (untreated oocytes). Previously, we reported that the fertility of delipated porcine IVM oocytes cryopreserved by our vitrification system was not different from that of undelipated fresh oocytes [13]. In this study, although we did not confirm the fertility of the delipated, vitrified oocytes, the low cleavage rate of the vitrified oocytes was probably a consequence of the reduction of developmental competence caused by the cryopreservation, not a reduction in fertility.

As shown in Table 1, the delipation from porcine IVM oocytes did not have a negative effect on the developmental competence with respect to the blastocyst formation rate. The present results raise the question of the roles of cytoplasmic droplets in porcine *in vitro* production (IVP) systems. Cytoplasmic lipid droplets in mammalian oocytes or embryos are thought to play important roles in energy metabolism during oocyte maturation, fertilization and early embryonic development [22]. On the other hand, the rate of nuclear maturation of porcine delipated GV-stage oocytes was not different from that of undelipated, fresh oocytes [8]. Moreover, it has been shown that the rate of development to the blastocyst stage and blastocyst cell number of parthenogenetic embryos at the four- to eight-cell stage following delipation are comparable to those of undelipated embryos [11]. Although the roles of lipid droplets in the cytoplasm for porcine IVP systems were not clarified in this study, their roles in porcine IVP are intriguing. Recently, it has been demonstrated that porcine MII-stage oocytes can be cryopreserved while retaining their ability to develop into fetuses [21]. In this study, no data were collected regarding the ability of embryos derived from delipated porcine IVM oocytes to develop into live piglets. Therefore, further investigation is required to evaluate the full-term development of embryos derived from such oocytes.

In conclusion, we have shown that delipated, vitrified porcine IVM oocytes can develop to the blastocyst stage following IVF; however, the blastocyst formation rate was low. Thus, further study is required to improve the cyrotolerance of delipated porcine IVM oocytes.

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