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—Full Paper—

Effect of Centrifugation Treatment before Vitrification on the Viability of Porcine Mature Oocytes and Zygotes Produced *In Vitro*

Tamás SOMFAI[1)#], Naomi KASHIWAZAKI[2)], Manabu OZAWA[1)], Michiko NAKAI[1)], Naoki MAEDOMARI[2)], Junko NOGUCHI[1)], Hiroyuki KANEKO[1)], Takashi NAGAI[3)] and Kazuhiro KIKUCHI[1)]

[1)]Division of Animal Sciences, National Institute of Agrobiological Sciences, Tsukuba 305-8602, [2)]Graduate School, Azabu University, Sagamihara 229-8501 and [3)]National Institute of Livestock and Grassland Science, Tsukuba 305-0901, Japan
[#]Present: National Livestock Breeding Center, Nishigo 961-8511, Japan

Abstract. The aim of the present study was to investigate the effects of centrifugation pretreatment on the viability and nuclear status of porcine *in vitro* matured (IVM) oocytes and on the developmental competence of *in vitro* fertilized (IVF) oocytes (zygotes) after cryopreservation by vitrification (Solid Surface Vitrification; SSV). Mature oocytes having the first polar body after IVM and zygotes having the second polar body at 10 h after IVF were centrifuged at 10,000 × g at 37 C for 20 min and then subjected to SSV. Their viability was evaluated by morphological appearance and fluorescein diacetate staining. The nuclear status of oocytes was evaluated 6 h after vitrification. The developmental ability to the blastocyst stage of vitrified zygotes was evaluated after 6 days of *in vitro* culture. Although centrifugation did not damage the oocytes directly, it drastically reduced the rate of live oocytes after SSV. The rates of vitrification-induced parthenogenetic activation were similar in both centrifuged and non-centrifuged oocytes (42.4 and 47.4%, respectively). Centrifugation had no significant effects on the viability of pronuclear oocytes. The development of vitrified zygotes to the blastocyst stage was significantly lower than that of the control irrespective of centrifugation pretreatment. There was no difference in the cleavage and blastocyst rates between the control and centrifuged zygotes after vitrification. There was also no difference in the total cell numbers of blastocysts between the control and centrifuged zygotes irrespective of vitrification. These results reveal that, in IVM porcine oocytes, centrifugation pretreatment is highly detrimental to cryotolerance; however, in zygotes, it has only a slight effect on viability and does not alter the developmental competence of surviving zygotes.

Key words: Centrifugation, Oocyte, Pig, Vitrification, Zygote

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The oocytes and early embryos of cattle and pigs are known to contain large numbers of cytoplasmic lipid droplets, which have an important role in their energy metabolism [1]. Nevertheless this characteristic has a huge impact on their availability for certain methods in animal biotechnology. For instance, it is known that a large number of lipid droplets causes the sensitivity of porcine embryos to low temperatures, which makes them very difficult to cryopreserve [2]. Also, the high lipid content that makes the cytoplasm of oocytes and early embryos dark blocks the visibility of organelles, which is essential for certain techniques such as microinjection of DNA into pronuclei [3].

Polarization of lipid droplets by centrifugation is an efficient method to increase visibility of pronuclei [4]. Besides its importance in the production of transgenic cattle and pigs, centrifugation can be also used to confirm the onset of oocyte activation by the existence of pronuclei [5] or to separate monospermic zygotes from polyspermic ones [6]. Moreover, after centrifugation treatment, polarized lipid can be removed mechanically using a suction pipette, which greatly improves the tolerance of early porcine embryos to chilling and freezing [2, 7]. Nevertheless, lipid removal using a micromanipulator is a rather time consuming procedure. Thus, only a limited number of embryos can be prepared per experiment using this method. On the other hand, without removal of polarized lipid, centrifugation has diverse effects on survival and developmental ability during freezing of mature bovine oocytes; although centrifugation has a negative effect on survival, it improves the developmental competence of the surviving oocytes [8]. To our knowledge, no study discussing the effect of lipid polymerization on survival or development of porcine oocytes is available. The sensitivity of porcine oocytes containing a large amount of lipids to cryopreservation procedures seems to be higher than that of bovine oocytes. As a consequence, cryopreservation of porcine oocytes by conventional slow cooling remains unsuccessful [9]. Some success in cryopreservation of immature and *in vitro* matured (IVM) porcine oocytes has been achieved by application of vitrification methods [10–12]. Despite recent improvements in survival rates, the developmental competence after vitrification of porcine IVM oocytes is still very poor [13, 14]. The vitrification procedure triggers parthenogenetic activation of oocytes at the metaphase-II (MII) stage, resulting in reduced fertilization rates during *in vitro* fertilization (IVF) and a reduced ability of the vitrified oocytes to form a male pronucleus after being penetrated by spermatozoa [14]. This phenomenon, therefore, greatly reduces the availability of vitrified MII oocytes for generation of viable porcine embryos. It is unclear weather the cooling-induced

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Correspondence: K. Kikuchi (e-mail: kiku@affrc.go.jp)

activation of oocytes is related to their lipid content, which is known to be responsible for the sensitivity of porcine embryos to chilling [2].

Centrifugation is widely used in laboratories to visualize pronuclei for selection of zygotes after IVF [5, 6, 15]; however, the potential effects of this procedure on the cryotolerance of porcine oocytes and zygotes have not been studied. The first objective of the present study was to investigate the effect of lipid polarization by centrifugation on the survival and nuclear status of IVM porcine oocytes cryopreserved by vitrification. Furthermore, to clarify whether centrifuged zygotes can be cryopreserved after pronuclear evaluation, the effect of centrifugation on the cryosurvival and *in vitro* developmental ability of fertilized oocytes was also studied.

Materials and Methods

Oocyte collection and IVM

Collection and IVM of porcine follicular oocytes were performed according to Kikuchi *et al*. [16]. Ovaries from prepubertal cross-bred gilts (Landrace × Large White) were collected at a local slaughterhouse and transported to the laboratory in Dulbecco's phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan) at 35–37 C within 1 h. Cumulus-oocyte complexes (COCs) were collected by scraping 3–6-mm follicles in a collection medium consisting of Medium 199 (with Hanks' salts; Sigma-Aldrich Chemical, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (Gibco Invitrogen, Carlsbad, CA, USA), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan) and antibiotics [100 units/ml penicillin G potassium (Sigma) and 0.1 mg/ml streptomycin sulfate (Sigma)]. Maturation culture was performed in a modified North Carolina State University (NCSU)-37 solution [17] containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 μM β-mercaptoethanol (Sigma), 1 mM dibutyryl cAMP (dbcAMP; Sigma), 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical, Tokyo, Japan) and 10 IU/ml hCG (Puberogen, 500 U; Novartis Animal Health, Tokyo, Japan) in 4-well dishes (Nunclon MultiDishes; Nalge Nunc International, Roskilde, Denmark) for 22 h under the tensions of 5% CO_2, 5% O_2 and 90% N_2 at 39 C. The COCs were subsequently cultured in the maturation medium without dbcAMP and hormones for an additional 24 h under the same gas conditions. Forty to fifty COCs were cultured in each well.

IVF and in vitro culture (IVC)

IVF and IVC were carried out according to Kikuchi *et al*. [16]. At the end of IVM, the COCs were transferred into 1 ml of the collection medium supplemented with 0.1% (w/v) hyaluronidase (Type-I, Sigma) for 30 sec, and then the oocytes were freed from the cumulus cells mechanically with a fine glass pipette in a hyaluronidase-free collection medium. The denuded oocytes were washed twice in the collection medium, and only those with a visible first polar body were separated under a stereomicroscope and used for IVF. The medium for IVF was a modified Pig-FM [18] supplemented with 2 mM caffeine and 5 mg/ml bovine serum albumin (BSA; Fraction V, Sigma). After washing three times in IVF medium, about 20 oocytes were transferred into 90 μl IVF droplets covered with paraffin oil (Paraffin Liquid; Nacalai Tesque, Kyoto, Japan). Frozen-thawed epididymal spermatozoa from a Landrace boar were preincubated in Medium 199 (with Earle's salts, pH adjusted to 7.8) for 15 min [19]. A small portion (10 μl) of sperm suspension was introduced into IVF medium containing oocytes, and they were then coincubated for 3 h at 39 C under 5% CO_2, 5% O_2 and 90% N_2. The final sperm concentration was 1×10^5 cells/ml. The day of IVF was defined as Day 0. After removal of spermatozoa from the surface of the zona pellucida by gentle pipetting with a fine glass pipette, IVC was performed in 500 μl drops of IVC-PyrLac and IVC-Glu on Days 0–2 and Days 2–6, respectively [16] in 4-well dishes in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39 C. The IVC-PyrLac consisted of NCSU-37 [17] without glucose but supplemented with 4 mg/ml BSA, 50 μM β-mercaptoethanol, 0.17 mM sodium pyruvate and 2.73 mM sodium lactate. The IVC-Glu was the original NCSU-37 containing 5.55 mM glucose and supplemented with 4 mg/ml BSA and 50 μM β-mercaptoethanol.

Centrifugation treatment

Mature or inseminated oocytes were placed in 700 μl of IVC-PyrLac containing 20 mM HEPES in 1.5 ml sterile eppendorf tubes and centrifuged at 10,000 × g at 37 C for 20 min in a microcentrifuge (VS-15000 CFN II; Vision, Tokyo, Japan). Cytochalasins were not used during centrifugation.

Vitrification

Oocytes and zygotes were cryopreserved by the Solid Surface Vitrification method originated by Dinnyes *et al*. [20], with some modifications [14]. Briefly, the oocytes or zygotes were washed three times in a base medium (BM; NCSU 37 supplemented with 20 mM HEPES and 0.4 mg/ml BSA) and then treated with equilibration medium [4% (v/v) ethylene glycol (EG, Sigma, E-9129) in BM] for 10 to 15 min at 37 C. The equilibrated oocytes/zygotes were washed in 20 μl droplets of vitrification solution [BM plus 35% (v/v) EG, 50 mg/ml polyvinyl pyrrolidone (Sigma, P-0930) and 0.3 M trehalose (Sigma, T-0167)], pipetted into a glass capillary in groups of 10–20 oocytes/zygotes and finally dropped with about 2 μl vitrification solution onto the cold dry surface of an aluminum foil floating on the surface of liquid nitrogen [13]. Rinsing and vitrification were performed in less than 30 sec. Vitrified droplets were warmed by transfer into a warming solution (0.4 M trehalose in BM) at 37 C using cooled forceps. One to 2 min later, the oocytes/zygotes were consecutively transferred for 1-min periods into 500-μl droplets of BM supplemented with 0.2 M, 0.1 M or 0.05 M trehalose. They were then washed three times in BM at 37 C. The vitrified-warmed oocytes and zygotes were transferred into 500-μl droplets of IVC-PyrLac and cultured *in vitro* as described above.

Evaluation of oocyte/zygote viability

Viability of oocytes was evaluated by both morphological examination and fluorescein diacetate (FDA) staining. Oocyte morphology was examined under a stereomicroscope (SZX12, Olympus, Tokyo, Japan). Oocytes with a normal spherical shape, evenly granular dark cytoplasm and a visible perivitelline space were considered to be live and were selected for further investiga-

tion. The live status of the selected oocytes was confirmed by FDA staining according to Mohr *et al*. [21]. Briefly, 5 mg/ml FDA (Sigma F-7378) stock solution was prepared in acetone. The stock solutions were kept in polypropylene tubes at –20 C for a maximum of three days. Staining solution was prepared by 2000 times dilution of FDA stock in PBS (supplemented with 5 mg/ml BSA) to obtain a final concentration of 2.5 μg/ml FDA. Oocytes were treated with this solution at 37 C for 2 min in a dark room and then washed three times in PBS and evaluated under a stereomicroscope with UV irradiation using a GFP-II filter (MZ16FA; Leica Microsystems, Heerbrugg, Switzerland). Live oocytes fluoresced a bright green colour. Viability of zygotes was evaluated by stereomicroscopic examination of morphology as described above.

Oocyte and embryo evaluation with orcein staining

For evaluation of the nuclear status of oocytes and counting of total cell numbers of blastocysts, the oocytes/embryos were mounted on glass slides and fixed with acetic alcohol (acetic acid: ethanol, 1:3) for at least 3 days, stained with 1% (w/v) orcein in acetic acid, rinsed in glycerol:acetic acid:water (1:1:3) and examined under a phase-contrast microscope with ×40 and ×100 objectives. Oocytes with a typical metaphase plate and one polar body (PB) were classified as normal metaphase-II (Normal MII). Oocytes having one polar body and abnormal nuclear morphology (dispersed chromosomes or a single cluster of condensed chromatin) were classified as "Abnormal MII". Oocytes with one PB and chromosomes showing the signs of sister chromatid segregation were classified as Anaphase-II (AII). Oocytes with one PB and segregated chromosomes showing the protrusion of the future second PB were classified as telophase-II (TII). Oocytes with two PBs and metaphase chromosomes were classified as metaphase-III (MIII). Because of their low frequency, AII, TII and MIII oocytes were grouped into one class (AII–MIII). Oocytes with a female pronucleus(ei) were classified as activated oocytes. In the present study, blastocysts were defined as having 10 or more cells with a clear blastocoele.

Experimental design

Experiment 1: The impact of vitrification with or without centrifugation on the viability and nuclear status of MII stage oocytes was evaluated in a 2 × 2 factorial design. After 44 h of IVM, oocytes at the MII stage were selected under a stereomicroscope according to the presence of the first polar body. Four treatment groups were investigated.

Group 1): Control oocytes were neither centrifuged, nor vitrified (centrifugation –/vitrification –).

Group 2): Oocytes were centrifuged but not vitrified (centrifugation +/vitrification –).

Group 3): Oocytes vitrified without centrifugation (centrifugation –/vitrification +).

Group 4): Oocytes were centrifuged and then vitrified (centrifugation +/vitrification +).

The viability of oocytes was evaluated after incubation in IVC-PyrLac medium for 6 h. The nuclear status of live oocytes was assessed after orcein staining.

Experiment 2: The effect of vitrification with or without centrifugation on the viability and *in vitro* developmental ability of zygotes was evaluated. After 44 h of IVM, oocytes were subjected to IVF and IVC. Oocytes with two polar bodies (considered as zygotes) were selected 10 h after IVF under a stereomicroscope. Zygotes were centrifuged, vitrified or vitrified after centrifugation. Control zygotes were neither centrifuged, nor vitrified. The viability of zygotes was evaluated 20 min after treatment based on their morphology. Live zygotes were cultured for 6 days as described above. Cleavage rates were recorded on Day 2 of IVC. Blastocyst rates were recorded on Day 6 of IVC. Cell numbers in blastocysts were evaluated after staining with orcein.

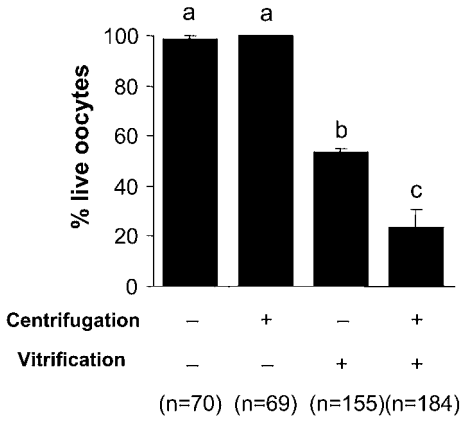

Fig. 1. Viability of porcine oocytes at the metaphase-II stage after different treatments at 44 h of *in vitro* maturation. Viability was evaluated by stereomicroscope according to the morphological appearance of the oocytes. Stereomicroscopic evaluation was verified by fluorescein diacetate staining. Total numbers of oocytes examined in each group (n values) are given in parentheses. [a–c]The percentages with different letters differ significantly (P<0.01).

Statistical analysis

Percentage data were subjected to an arcsine transformation and analyzed by one-way ANOVA using the KyPlot package (Ver. 4.0, KyensLab, Tokyo, Japan). Three replicates of Experiment 1 and six replicates of Experiment 2 were performed.

Results

Experiment 1

The viability of all oocytes selected by morphology was verified by FDA staining. As shown in Fig. 1, centrifugation treatment alone did not affect the viability of MII oocytes; the percentages of live oocytes in the non-vitrified groups with or without centrifugation were very similar (100 and 98.6%, respectively). The percentage of live oocytes after vitrification of non-centrifuged oocytes was significantly reduced compared with the control (P<0.01; 53.4 and 98.6%, respectively). When oocyte vitrification was combined with centrifugation, the rate of live oocytes was significantly lower than that for oocytes after vitrification without centrifugation (23.4 and 53.4%, respectively).

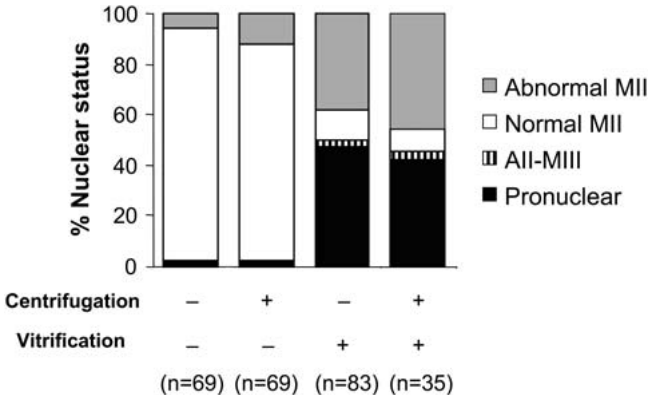

Fig. 2. Nuclear statuses of vitrified/warmed oocytes after *in vitro* culture for 6 h. Total numbers of oocytes examined in each group are given in parentheses. AII-MIII: oocytes at the stage of anaphase-II, telophase-II or metaphase-III; MII: metaphase-II. Oocytes having one polar body and abnormal nuclear morphology (dispersed chromosomes or a single cluster of condensed chromatin) were classified as "Abnormal MII". The percentages of Normal MII, Abnormal MII and Pronuclear oocytes differ significantly (P<0.01) between the vitrified and non-vitrified groups. The percentages of oocytes did not differ significantly between the centrifuged and non-centrifuged groups in the vitrified and non-vitrified groups. Three replicated trials were carried out.

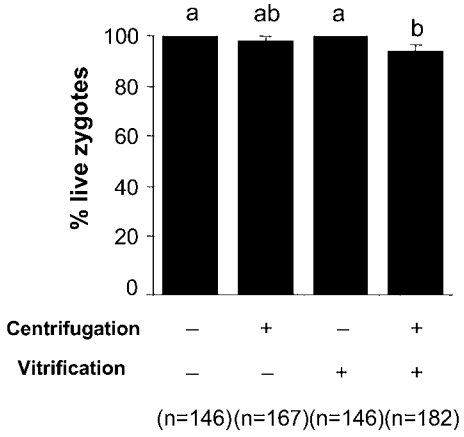

Fig. 3. Viability of porcine zygotes after different treatments at 10 h after *in vitro* fertilization. Viability of zygotes was evaluated by stereomicroscopic observation of zygote morphology. Total numbers of zygotes examined in each group are given in parentheses. [a, b]The percentages with different letters differ significantly (P<0.05).

Table 1. *In vitro* development of fertilized oocytes with two polar bodies after centrifugation and/or vitrification 10 h after IVF

Treatment		Total	Cleaved	Blastocyst	Total cells/
Centrifugation	Vitrification		(%)	(% total)	blastocyst
–	–	126	95 (75.2 ± 2.8)	29 (24.5 ± 4.4)[a]	45.2 ± 1.5
–	+	142	101 (70.8 ± 5.8)	13 (9.2 ± 1.8)[b]	45.1 ± 4.0
+	–	126	94 (76.1 ± 6.5)	26 (20.5 ± 6.2)[a]	42.2 ± 7.2
+	+	148	98 (66.5 ± 5.9)	8 (5.2 ± 1.6)[b]	46.4 ± 7.2

[a,b] Percentages with different letters differ significantly (P<0.05) in the same column. Five replicates of the experiment were performed.

The nuclear status of non-vitrified oocytes with or without centrifugation treatment did not differ significantly; in these groups, most oocytes were at the MII stage 6 h after treatment (87.5 and 91.7%, respectively; Fig. 2). After vitrification, however, a significantly higher proportion of oocytes were at the pronuclear stage in both the non-centrifuged and centrifuged groups (P<0.01; 47.4 and 42.4%, respectively) compared with both groups of non-vitrified oocytes (0 and 2.6%, respectively). The percentages of oocytes at the anaphase-II/telophase-II or metaphase-III stages (jointly referred to as the AII-MIII stage) were not significant among the groups. Significantly higher rates of non-pronuclear vitrified oocytes in the non-centrifuged and centrifuged groups had abnormal nuclear morphology, such as dispersion or cluster formation of condensed chromosomes (P<0.01; 38.1 and 45.7%, respectively), compared with both groups of non-vitrified oocytes (5.5 and 11.9%, respectively). There was no significant difference in the distribution of pronuclear, AII-MIII, MII and abnormal MII oocytes between the centrifuged and non-centrifuges groups after vitrification (Fig. 2).

Experiment 2

There was no significant difference in the rate of viable zygotes between the control and vitrified groups without centrifugation (100 and 98.4%, respectively). Centrifugation treatment did not modify zygote viability; however, when vitrified after centrifugation pretreatment, the rate of live zygotes was slightly but significantly reduced (P<0.05; 100 *vs.* 94.7%, respectively; Fig. 3).

When surviving zygotes were cultured *in vitro*, there was no significant difference in the rate of cleaved embryos between the control and vitrified groups irrespective of centrifugation pretreat-

ment (Table 1). The blastocyst rates of non-centrifuged and centrifuged zygotes were, however, significantly lower ($P<0.05$; 9.2 and 5.2%, respectively) after vitrification compared with those of the control groups (24.5 and 20.5%, respectively; Table 1). Centrifugation pretreatment had no effect on the blastocyst rates of control and vitrified zygotes. There was no significant difference in the total cell numbers of blastocysts between the treatment groups (42.2–46.4 cells/blastocyst; Table 1).

Discussion

Our results concerning the viability of vitrified MII oocytes revealed that, although centrifugation did not harm the oocytes directly, it drastically reduced the cryotolerance of IVM porcine oocytes. This confirms previous results for IVM bovine oocytes reported by Otoi *et al*. [8]. It is well known that the primary site of cryopreservation-induced injuries in porcine oocytes is the membrane [22]. It is plausible that after centrifugation those areas of the oolemma that are associated with the polarized lipid become highly sensitive to cooling. Nevertheless, unlike matured oocytes at the MII stage, centrifugation pretreatment of porcine early zygotes vitrified at 10 h after IVF had only a very slight effect on their subsequent viability and development *in vitro*. Our results clearly indicate the higher cryotolerance of pronuclear oocytes compared with that of unfertilized MII stage oocytes. Similarly, in cattle, the post-thaw survival rates of frozen zygotes have been found to be higher than those of unfertilized oocytes [23]. Supposedly, some of the changes that occur in oocytes during the first 6–8 h of fertilization greatly increase the tolerance of the oocyte membrane to low temperatures. During fertilization and early development, oocytes undergo several structural and ultrastructural changes that can possibly affect their cryosensitivity. Recently, a significant difference in phase-transition temperatures (the temperature at which the membrane phospholipids transform from a liquid crystalline phase to a crystal gel phase) was found between the human oocyte and zygote stages, suggesting differences in their phospholipid compositions and cholesterol concentration [24]. Thus, it is very likely that the changes in membrane characteristics during fertilization and early development are responsible for the improved cryotolerance of zygotes. The lipid content and composition, which have a huge impact on the cryotolerance of oocytes, may also differ between oocytes and zygotes. For instance, bovine 2-cell stage embryos have been reported to contain significantly fewer lipids than unfertilized oocytes [25]. Although the total triglyceride content does not seem to change during fertilization and early development of porcine oocytes [26], the number and size of lipid droplets in pronuclear oocytes appear to be smaller than those in unfertilized MII stage oocytes [27]. Changes in lipid characteristics may also contribute to improvement of cryoresistance during pronuclear formation. However, it must be noted that, in the present study, there were some differences in the methodologies used to evaluate the viability of oocytes and zygotes; viability of oocytes was evaluated by stereomicroscopic observation followed by FDA staining, whereas viability of zygotes was evaluated by only stereomicroscopy. The reason why we omitted FDA staining of zygotes was the detrimental effect of this staining method on *in vitro* development of porcine zygotes (Somfai *et al*., unpublished observation). Nevertheless, in Experiment 1, all oocytes selected by stereomicroscopic evaluation were proven to be alive by FDA staining. Therefore, we concluded that the viability data from Experiment 1 (oocyte) and Experiment 2 (zygote) could be compared.

Vitrification has been reported to trigger parthenogenesis in MII oocytes in sheep [28] and pigs [14]. However, little is known about the exact mechanism of cryopreservation-induced parthenogenetic activation of oocytes. Activation of mammalian oocytes is known to be characterized by elevation of the Ca^{2+} concentration in the cytoplasm [29]. The most likely reason for parthenogenetic activation of cryopreserved oocytes is therefore the rise in cytoplasmic Ca^{2+} concentration caused by the vitrification procedure. It has also been reported that treatment of mouse oocytes with cryoprotectants such as DMSO and EG induces an increase in the intracellular calcium concentration solely (DMSO) or partially (EG) from internal calcium pools causing zona hardening, which refers to the onset of oocyte activation [30]. Similarly, treatment of sheep oocytes with DMSO or EG results in their parthenogenetic activation [31]. In our previous study, however, treatment with cryoprotectants did not induce parthenogenesis, whereas more than 40% of vitrified oocytes were activated and formed a female pronucleus 6 h after treatment [14]. Therefore, it seems that, in pigs, it is rather the cooling procedure and not the treatment with cryoprotectant(s) that triggers parthenogenetic activation. Supporting this theory, cooling to 14 C has been reported to induce elevation of intracellular calcium levels by mobilizing Ca^{2+} from intracellular stores through ryanodine receptors [32]. In our recent experiments, we found that vitrification triggers similar frequency of parthenogenetic activation of IVM porcine oocytes not only in Ca^{2+}-containing but also Ca^{2+}-free media (Somfai *et al*., unpublished data). Therefore, it is probable that the cryopreservation procedure mobilizes Ca^{2+} from intracellular calcium stores. The main calcium store of mammalian oocytes is known to be the endoplasmic reticulum (ER) [33]. In the pig, Ca^{2+} is normally mobilized from the intracellular stores through both inositol 1,4,5-triphosphate and the ryanodine receptors [34]. Clusters of mitochondria placed in close proximity to the ER membrane also play an important role in regulation of the intracellular Ca^{2+} level [35]. In bovine and porcine oocytes, lipid droplets are associated with a smooth ER and mitochondria forming metabolic units [27, 36–38]. Cryopreservation is known to cause an extensive decrease in the number and activity of mitochondria in bovine and porcine oocytes [39, 40]. This raises the question of whether there are any relationships between the association of ER and mitochondria with lipid droplets and cooling-induced parthenogenetic activation in porcine oocytes. Centrifugation successfully separates the ER and mitochondria from lipids in porcine, ovine and cattle oocytes [4, 41]. Our results concerning the oocyte nuclear status revealed that vitrification triggered parthenogenesis of oocytes irrespective of centrifugation pretreatment. This suggests that cooling-induced oocyte activation is not related to the presence of lipid droplets in the cytoplasm. Further studies are needed to clarify and prevent the mechanism of vitrification-induced oocyte activation.

To maintain the polarized lipid in the periphery of cells, centrif-

ugation can be combined with cytochalasin B (CB) treatment [2]. Besides treatment of oocytes with actin polymerization, inhibitors such as CB or cytochalasin D have some beneficial effects on the cryotolerance of porcine oocytes and embryos [10, 12, 42]. Nevertheless, treatment of MII oocytes before fertilization with CB may also have some disadvantages, since inhibition of actin polymerization is known to reduce the ability of oocytes to be fertilized by sperm and to extrude the second polar body [43]. Moreover, CB may also be detrimental to embryos, since this drug is known to block glucose transporters during early embryo development [44]. Therefore, we omitted use of CB during lipid polarization.

To our knowledge, this is the first paper to describe successful cryopreservation of porcine zygotes to obtain blastocysts. The rate of vitrified zygotes that developed to the blastocyst stage was lower than that of the control; however, it should be noted that there was no difference in quality (cell numbers) between the blastocysts developed from vitrified and control zygotes. We believe that, by neglecting polyspermic oocytes, the efficiency of zygote vitrification can be increased.

In conclusion, centrifugation pretreatment was found to be highly detrimental to the cryotolerance of IVM porcine oocytes; however, it had only a very slight effect on the survival of zygotes without affecting their developmental competence. Therefore, we believe that our centrifugation protocol can be used to select monospermic zygotes for cryopreservation.

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