Cryopreservation of Immature Porcine Oocytes Using Open Pulled Straw Vitrification

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Abstract

The aims of this study were to investigate the meiotic competence of immature porcine oocytes following open pulled straw (OPS) vitrification and warming and also to test the efficiency of the pretreatment of immature oocytes with CB prior to OPS vitrification. Experiment 1, cumulus oocyte complexes (COCs) were randomly assigned to OPS vitrification, cryoprotectant (CPA) toxicity test and non-CPA/non-vitrified controls. Experiment 2, the COCs were treated with 7.5 μg/ml CB for 30 min. The oocytes were either immediately fixed or further incubated in a CB-free medium for 5 h, non-CB treated COCs served as controls. Experiment 3, CB-pretreated COCs were vitrified. The COCs exposed to CB and vitrification solutions and non-CPA, non-CB treated oocytes served as controls. In all cases, all the COCs were matured *in vitro* for 40-44 h. After IVM, the oocytes were examined for the stages of nuclear maturation. Vitrifying and warming significantly reduced the capability of the immature porcine oocytes to resume and reach metaphase (M II) (21.7%), compared unfavorably with the control (81.8%, $p<0.05$). This, however, was not the result from the cryoprotactants *per se*, since the number of CPA-treated oocytes reaching MII stage did not significantly differ from the control (76% vs. 81.8%, $p>0.05$). While cytochalasin B depolymerized actin cytoskeleton, this effect was completely reversible during 5 h of culture. Pretreatment with CB before the vitrification of immature porcine oocytes significantly yielded greater MII rates compared with non-CB oocytes (34.9% vs. 21.7%). In conclusion, porcine immature oocytes can be cryopreserved by OPS vitrification but it adversely affects the meiotic competence. Cytochalasin B reversibly depolymerizes actin microfilaments and improves the cryopreservability of porcine immature oocytes.

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บทคัดย่อ

การแช่แข็งโอโอไซต์สุกรระยะไม่พร้อมปฏิสนธิด้วยวิธี Open Pulled Straw วิทริฟิเคชั่น

ธีรวัฒน์ ธาราศานิต ศศิธร รุ่งอรุณเลิศ มงคล เตชะกำพุ[*]

การศึกษานี้มีวัตถุประสงค์เพื่อตรวจความสามารถในการแช่แข็งโอโอไซต์สุกรด้วยวิธี Open Pulled Straw Vitrification (OPS) และทดสอบประสิทธิภาพของไซโตคลาลาซิน-บี (CB) ต่ออัตราความสำเร็จในการแช่แข็ง โดยแบ่งเป็น 3 การทดลอง การทดลองที่ 1 ศึกษาประสิทธิภาพการแช่แข็งด้วยวิธี OPS และความเป็นพิษของสารป้องกันการแช่แข็ง เปรียบเทียบกับกลุ่มควบคุม การทดลองที่ 2 ศึกษาผลของ CB ต่อการเปลี่ยนแปลงโครงสร้างของแอคติน ไมโครฟิลาเมนต์ โดยทำการแช่โอโอไซต์ในสารละลายที่มี CB ที่ความเข้มข้น 7.5 ไมโครกรัมต่อมิลลิลิตร นาน 30 นาที จากนั้นทำการตรึงโอโอไซต์ทันที หรือทำการเลี้ยงโอโอไซต์ต่ออีก 5 ชั่วโมง การทดลองที่ 3 ทดสอบผลของ CB ต่อความสำเร็จในการแช่แข็งแบบ OPS โดยแช่โอโอไซต์ในสารละลายที่เติม CB ก่อนจะนำไปทำการแช่แข็ง และเปรียบเทียบกับกลุ่มควบคุม   ในทุกการทดลองเลี้ยงโอโอไซต์ให้เจริญพร้อมปฏิสนธินาน 40-44 ชั่วโมง จากการทดลองพบว่าการแช่แข็งด้วยวิธี OPS และการทำละลายมีผลลดความสามารถการเจริญพร้อมปฏิสนธิ (MII) ของโอโอไซต์อย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม (ร้อยละ 21.7 และ 81.8 ตามลำดับ, *p*<0.05) โดยผลดังกล่าวไม่มีสาเหตุจากสารป้องกันการแช่แข็ง เนื่องจากจำนวนโอโอไซต์ที่เจริญถึงระยะพร้อมปฏิสนธิไม่แตกต่างจากกลุ่มควบคุม (ร้อยละ 76 เทียบกับ 81.8, *p*>0.05) การเติม CB มีผลทำให้เกิดการแยกตัวของแอคติน ไมโครฟิลาเมนต์ ที่ซึ่งสามารถกลับคืนสภาพได้อย่างสมบูรณ์ภายใน 5 ชั่วโมง ของการเลี้ยงโอโอไซต์ การเติม CB ก่อนแช่แข็งโอโอไซต์ทำให้อัตราการเจริญพร้อมปฏิสนธิเพิ่มขึ้นเมื่อเทียบกับกลุ่มที่ไม่ได้เติม CB (ร้อยละ 34.9 และ 21.7 ตามลำดับ) การศึกษาครั้งนี้สรุปได้ว่าสามารถทำการแช่แข็งโอโอไซต์สุกรระยะไม่พร้อมปฏิสนธิได้ด้วยวิธี OPS แต่มีผลในทางลบต่อการเจริญพร้อมปฏิสนธิ การเติม CB มีผลทำให้เกิดการแยกตัวของแอคติน ไมโครฟิลาเมนต์และเพิ่มประสิทธิภาพในการแช่แข็งโอโอไซต์สุกรระยะยังไม่พร้อมปฏิสนธิ

คำสำคัญ : ไซโตคลาลาซิน-บี การเจริญพร้อมปฏิสนธิ โอโอไซต์ โอ พี เอส วิทริฟิเคชั่น สุกร

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Introduction

The cryopreservation of oocytes plays an important part in assisted reproduction techniques for both research and clinical applications because this technique allows 'long-term' genetic preservation and related experiments can be performed at a convenient time. To date, oocyte cryopreservation has been used to treat infertility problems in woman and to salvage the genetic potential of valuable wild species (Porcu, 2001; Smith and Silva, 2004). However, while live offspring have been produced from a wide range of species, the overall success of oocyte cryopreservation in pig, by means of developmental competence, is currently poor. In this regard, several strategies have been examined aimed at improving the cryopreservability of porcine oocytes and more specifically to reduce cryoinjury during freezing and thawing. Oocyte has a structural complexity with poor plasma membrane permeability to water and cryoprotectants. In addition, porcine oocytes contain high amounts of lipid within the ooplasm (McEvoy et al., 2000) and this has been postulated to decrease the cryopreservability of the oocytes since removal of lipid (delipidation) within the oocyte prior to freezing improves oocyte's quality post-thawing (Nagashima et al., 1994). Until recently, the cryopreservation of immature porcine oocytes has been reported with varying degrees of success. For example, 2.8 to 46.8% of cryopreserved-thawed germinal vesicle stage (GV) oocytes resumed and reached metaphase II stage *in vitro* (Isachenko et al., 1998; Fujihira et al., 2004; Hara et al.,

2005; Park et al., 2005; Varga et al., 2006). Vitrification, a cryopreservation technique that uses the combination of a high concentration of cryoprotectants and an extremely fast freezing rate, has been an increasingly useful means of reducing cryoinjury during cryopreservation. To date, several vitrification techniques for porcine oocytes and embryos have been examined, such as solid surface vitrification (Gupta et al., 2007), microdroplet (Misumi et al., 2003) and minimum volume cooling (Esaki et al., 2004) techniques. In addition, the open pulled straw (OPS) vitrification developed by Vajta et al. (1998) has been widely used to cryopreserve oocytes from a number of species. A thin-walled OPS straw and a small volume of cryoprotectants facilitate a remarkable increase in the cooling rate (a theoretical rate of 20,000°C/min.) compared favorably to 2,500°C/min. of conventional straw.

Cytochalasin B (CB), a reversible actin depolymerizer, has been shown to reduce cytoskeleton damage during cryopreservation. and, thereby, improving the viability of frozen-thawed porcine embryos (Dobrinsky et al., 2000). The addition of CB has also been demonstrated to decrease cryoinjuries during vitrification and the warming of immature oocytes (Fujihara et al., 2004; Isachenko et al., 1998).

The aims of this study were to investigate the meiotic competence of immature porcine oocytes following open pulled straw (OPS) vitrification and warming and also to test the efficiency of the pretreatment of immature oocytes with CB prior to OPS vitrification.

Materials and Methods

Isolation of cumulus-oocyte complexes (COCs): Porcine ovaries were collected from a local abattoir and transported to the laboratory within 2 h. Upon arrival, the ovaries were washed twice in 0.9% (w/v) saline solution supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. The COCs were aspirated from 3-6 mm follicles with an 18-gauge needle attached to a 10-ml syringe into a 50-ml conical tube. To collect the COCs, the follicular contents containing the COCs were allowed to settle for 5 min, and the sediment was then collected and washed twice in Hepes buffered Tyrode's medium supplemented with 0.1% (w/v) polyvinyl alcohol (TL-Hepes-PVA). Only uniform-sized COCs, with more than two layers of intact cumulus cell investment, were selected under a stereo microscope (x40, Nikon SM 2645, Japan).

Vitrification and warming of oocytes: The open pulled straw (OPS) vitrification procedure has been described by Vajta et al. (1998) with minor modifications. All manipulations were performed at 38.5°C on warm plate (MP10DM, Kitazato, Japan). The COCs designated for OPS vitrification were maintained in a holding medium (HM) which consisted of Hepes buffered TCM-199, 20% fetal calf serum (FCS) and 100 IU/ml penicillin and 100 µg/ml streptomycin. In the case of the pretreatment of the COCs with CB, the COCs were incubated with 7.5 µg/ml CB in HM for 30 min prior to OPS vitrification.

OPS vitrification was performed by transferring approximately 10 COCs to a 100µl droplet of vitrification solution I (VS I) containing 10% (v/v) dimethyl sulphoxide (DMSO) and 10% (v/v) ethylene glycol (EG) in HM for 30 sec. Subsequently, the COCs were immersed in vitrification solution II (VS II) containing 20% (v/v) DMSO, 20% (v/v) EG, and 0.5 M sucrose. Approximately 2 µl of VSII and the COCs were loaded into OPS and finally plunged into liquid nitrogen. The COCs were exposed to VSII for 25 sec.

For thawing, the vitrified COCs were removed from the liquid nitrogen and warmed by submerging the OPS straw immediately into a warming solution (37°C) consisting of HM supplemented with 0.3 M sucrose. The vitrified-warmed COCs were then incubated in this warming medium for 5 min and then in HM without sucrose for 5 min.

Cryoprotectant toxicity test: Because the high concentration of cryoprotectants used for vitrification is likely to be toxic to the oocytes, the probable toxicity of cryoprotectants was therefore examined. This was performed by equilibrating the COCs with VS I and VS II

as described above. The cryoprotectant-treated COCs were subsequently incubated for 5 min in warming solution and then finally in HM for 5 min prior to IVM.

***In vitro* maturation of oocytes:** The COCs subjected to *in vitro* maturation (IVM) were washed twice in IVM basic medium, consisting of tissue culture medium-199 (TCM-199), 10% (v/v) porcine follicular fluid, 100 IU/ml penicillin and 100 μg/ml streptomycin. The two-step IVM was performed at 38.5°C for 40-44 h in a humidified condition of 5% CO_2 in air. For the first 24 h, the COCs were cultured in a maturation medium supplemented with 10 IU/ml pregnant mare's serum gonadotropin and 10 IU/ml human chorionic gonadotropin. Subsequent culture was carried out on the same IVM basic medium for an additional 16-20 h without hormone supplementation.

Assessing the stage of nuclear maturation: After 40-44 h of IVM, the cumulus cells were completely removed from the oocytes by vortexing in 1% (w/v) trypsin solution. Denuded oocytes were then fixed in 4% (w/v) paraformaldehyde overnight at 4°C for a further analysis. To determine the nuclear stages of the oocytes, the denuded oocytes were washed twice in PBS supplemented with 0.1% (w/v) BSA and then stained with fluorescent DNA labeling, 4'-6-Diamidino-2-phenylindole (DAPI), for 10 min at room temperature. The fluorescently labeled oocytes were subsequently mounted on a glass microscopic slide and examined using an epifluorescent microscope (TE300, Nikon, Japan). The stages of the nuclear maturation of the porcine oocytes were classified as has been described elsewhere. Briefly, immature fully grown oocytes or germinal vesicle stage (GV) were typified by a confined chromatin that envelops with an intact nuclear membrane, while metaphase I (MI) oocytes contained two sister chromatids aligned as the metaphase plate. The metaphase (MII) oocytes were classified by the oocytes contained with the metaphase plate and the first polar body that had been extruded. Degenerate oocytes demonstrated a loss of plasma membrane integrity and/or showed either scattered or dispersed chromatin configuration.

Immuno-labeling of actin microfilaments: The oocytes destined for immuno-labeling of the actin microfilament were denuded and fixed overnight in 4% (w/v) paraformaldehyde. The oocytes were subsequently washed twice in PBS supplemented with 0.1% (w/v) bovine serum albumin and incubated with 0.165 μM Alexa Fluor® 488 phalloidin (Invitrogen, The Netherlands) for 30 min. The fluorescently labeled oocytes were then mounted on glass microscopic slides in a 2 μl droplet of antifade medium (Vetashield, Vector Lab, CA, USA) to retard photobleaching and sealed under a coverslip using nail polish. Examination was performed with an epifluorescent microscope (BX51, Olympus, Japan) equipped with digital camera (E410, Olympus, Japan). The actin cytoskeleton was therefore classified as grade I when the oocytes displayed a complex network of tread-like microfilaments distributed throughout the ooplasm, while grade II oocytes were typified by the disappearance, dispersal or multifocal clump patterns of actin microfilaments (Figure 1). All chemicals were purchased from Sigma-Aldrich St. Louis, USA, unless otherwise indicated.

Experimental design

Experiment 1 examined the effect of OPS vitrification on the cryopreservability of immature porcine oocytes, in terms of the success of *in vitro* maturation. The COCs were randomly assigned to one of three treatments: OPS vitrification and warming (n=240); a cryoprotectant toxicity test (n=125) and non-treated control (n=170). Subsequently, all COCs were matured *in vitro* for 40-44 h.

Experiment 2 examined the effect of cytochalasin B on the actin microfilament depolymerization of the oocytes. The COCs were treated with 7.5 μg/ml cytochalasin B for 30 min, and the oocytes were either immediately fixed (n=54) or further incubated in CB-free IVM medium for 5 h (n=42) in order to test the reversibility
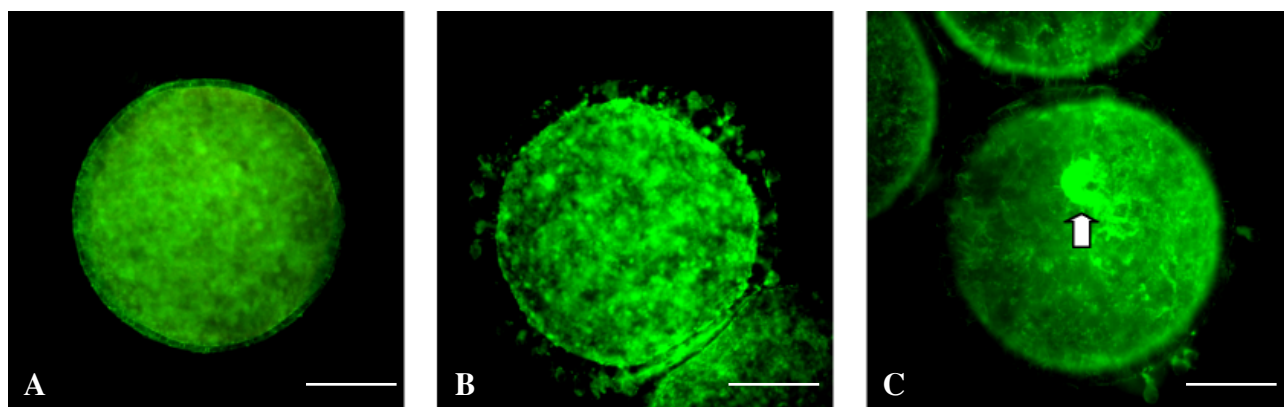
Figure 1 Photomicrographs demonstrating normal (grade I) and depolymerized-actin (grade II) oocytes. Non-cytochalasin B treated oocyte demonstrates a network of actin cytoskeleton typically distributed throughout the ooplasm (A) and clumping of actin microfilaments (arrow) of oocytes treated with cytochalasin B for 30 min (B and C). Scale bars represent 50 μM.

of cytochalasin B. Forty-one non-CB treated COCs served as controls.

Experiment 3 tested whether the pretreatment of immature oocytes with cytochalasin B prior to vitrification would really improve the cryopreservability of immature porcine oocytes. Cytochalasin B treatment was performed essentially as described in experiment 1 and 2. The COCs (n=106) were treated with cytochalasin B to induce the depolymerization of actin microfilaments prior to OPS vitrification. The COCs exposed to CB and vitrification solutions (n=42) and non-CPA, non-CB treated oocytes served as controls (n=140).

Statistical analysis: For analysis, the oocytes matured *in vitro* were classified as the germinal vesicle (GV) stage, metaphase I (MI) and metaphase II (MII). The oocytes that had dispersed or absent chromatin within the ooplasm were classified as degenerate oocytes. The data used for statistical analysis was pooled from independent 3-4 experimental replications. The Chi-square test was used to compare the stage of maturation among experimental groups. Non-parametic tests (Kruskal-Wallis and Mann-Whitney U) were used to compare the actin cytoskeleton patterns. In all cases, the statistic tests were performed using SPSS 15.0 for windows (SPSS Inc., Chicago, IL, USA). Difference was considered significant when $p < 0.05$.

Result

While the *in vitro* culture system used in this study was sufficient to induce meiotic competence, in terms of the oocytes that resumed and reached MII stage after 40-44 h of *in vitro* maturation (MII rate= 81.8%), vitrifying and warming immature porcine oocytes significantly reduced the proportion of oocytes reaching MII stage (21.7%) compared to the controls ($p<0.05$). Since complete vitrification of solutes essentially requires a high concentration of cryoprotectants, the immature porcine oocytes were exposed to the cryoprotectants used for vitrification in order to test the cryoprotectant toxicity. Indeed, the cryoprotectants (EG and DMSO) *per se* did not affect the meiotic competence of the immature porcine oocytes, because the MII rate (76%) of cryoprotectant treated immature oocytes did not significantly differ from the control (Table 1).

Actin microfilaments were typically distributed throughout the ooplasm of the germinal vesicle stage oocyte and with a slightly greater concentration beneath the oolema (Figure 1A). When immature porcine oocytes were treated with 7.5 μg/ml CB, a large number of these oocytes demonstrated a dispersal pattern (grade II) of actin microfilaments, which was characterized by large areas or formation as intracytoplasmic clumps (Figure 1B, 1C, Table 2) indicating CB-induced actin

Table 1   The meiotic competence of immature porcine oocytes following vitrification (OPS), warming and *in vitro* maturation.

Group	Oocytes (n)	Stage of nuclear maturation (%)				
		GV	MI	MII	Degenerate	Unidentifiable morphology
OPS	240	71 (29.6)[a]	54 (22.5)[a]	52 (21.7)[a]	62 (25.8)[a]	1 (0.4)
CPA toxicity test	125	2 (1.6)[b]	17 (13.6)[b]	95 (76)[b]	9 (7.2)[b]	2 (1.6)
Control	170	4 (2.4)[b]	18 (10.6)[b]	139 (81.8)[b]	5 (2.9)[b]	4 (2.4)

[a,b]Within a column, values with different superscripts denote significant difference ($p<0.05$)

Table 2   The patterns of actin microfilaments within oocytes treated with cytochalasin B.

Group (n)	Stage of nuclear maturation	
	grade I (%)	grade II (%)
Non-CB treated oocytes (n = 41)	38 (90.5)[a]	3 (9.5%)[a]
CB treated oocytes (n = 54)	12 (22.2)[b]	42 (77.8)[b]
CB treated oocytes + 5 h IVM (n = 42)	38 (90.5)[a]	4 (9.5)[a]

[a,b]Within a column, values with different superscripts denote significant difference ($p < 0.05$)

Table 3   The effect of cytochalasin B (CB) on the nuclear maturation of vitrified-warmed immature porcine oocytes.

Group	Oocytes (n)	Stage of nuclear maturation (%)			
		GV	MI	MII	Degenerate
Control + CB	140	5 (3.6)[a]	7 (5)[a]	123 (87.9)[a]	5 (3.6)[a]
CPA + CB	42	3 (7.1)[a]	1 (2.4)[a]	36 (85.7)[a]	2 (4.8)[a]
OPS + CB	106	14(13.2)[b]	34 (32.1)[b]	37 (34.9)[b]	21 (19.8)[b]

[a,b]Within a column, values with different superscripts denote significant difference ($p < 0.05$).

depolymerization. The actin microfilaments, however, were repolymerized similar to non-CB treated oocytes after *in vitro* culture for 5 h in 'CB-free' IVM medium.

Experiment 3 examined the effect of cytochalasin B on the capability of immature porcine oocytes to withstand vitrification and warming. COCs treated with CB prior to vitrification significantly improved the *in vitro* maturation rate (34.9%) compared to non-CB treated oocytes (21.7%, Table 1), although this was significantly lower than those of the control (87.9% MII) and cryoprotectant/CB treated oocytes (85.7% MII, Table 3).

Discussion

This study examined the cryopreservability of immature porcine oocytes and the effect of pretreatment with cytochalasin B prior to OPS vitrification on the success of *in vitro* maturation. To date, the overall success of oocyte cryopreservation has been remarkably limited, principally because the oocyte has a poor plasma membrane permeability to water and to cryoprotectants (Leibo, 1980). Some vital structures of oocytes such as plasma membrane (Ashwood-Smith et al., 1988), mitochondria (Rho et al., 2002) and microtubule (Saunders and Parks, 1999) are prone to damage during

cryopreservation. Irreversible cryoinjury of the oocyte occurring during freezing and thawing is often associated with an increase of intracellular ice formation during cryopreservation that, as a result, causes cell death or poor developmental capability. To minimize the incidence of intracellular ice formation, vitrification techniques has been developed. Indeed, although this vitrification technique has been known to reduce lethal intracellular ice formation within the cytoplasm, vitrification (solidification of solutes into a glass-like structure) occurs only when the cooling rate is reduced extremely fast (for review see: Liebermann et al., 2002). More importantly, this technique also requires a high concentration of cryoprotectant (s) which often becomes toxic.

Our data demonstrates that immature porcine oocytes can be frozen using the open pulled straw vitrification technique, although the outcome obtained in this study was far less than those results reported in other species such as mouse and bovine. For instance, vitrified-warmed immature bovine oocytes using OPS technique yielded 60% MII rates (Hurtt et al., 2000). It is thus clearly indicated that porcine oocytes are fairly sensitive to cryopreservation. To date, the exact causes of the poor freezability of porcine oocytes has not yet been well understood but it has been hypothesized that high lipid contents within the porcine oocyte appear to impede the transient movement of cryoprotectant and water across the oolema since delipidation of oocytes improves the cryopreservability of the oocytes (Hara et al., 2005; Park, et al., 2005). It should be noted, however, that this technique appears to affect the subsequent viability of the embryo *in utero* (Nagashima et al., 1994, 1995).

Because OPS vitrification preserves the chromosomal pattern and meiotic spindle morphology better than conventional straw (Chen et al., 2000) and given that meiotic spindle/microtubule plays an essential role during fertilization and embryo development, the cryopreservation of immature oocytes has therefore become an attractive issue (Shaw, et al., 2000). The immature oocytes are quiescent and the chromosomes

are condensed and enveloped by the nuclear membrane. More importantly, a cold sensitive meiotic spindle of immature oocytes has yet to be formed. In this study, OPS vitrification of immature oocytes reasonably yielded a maturation rate of 21.7% which was greater than other reports (MII rates of 2. 8% and 6.1%). This likely involves the types of cryoprotectants and also the vitrification techniques (Isachenko et al., 1998; Rojas et al., 2004; Varga et al., 2006), probably because a combination of a thin-walled straw and a high concentration of cryoprotectants facilitates a rapid reduction of the freezing rate that, theoretically, reduces cryoinjury. Despite controversial success, the vitrification of oocytes after *in vitro* maturation has been demonstrated to be an acceptable development so that Rojas et al. (2004) reported that MII stage porcine oocytes tolerated cryopreservation better than immature stage oocytes as well as in other species (Hurtt, et al., 2000), although the chromosomal disjunction of the MII oocyte possibly occurs during cooling, resulting in aneuploidy of the embryo.

In addition to freezing techniques, suboptimal cooling and freezing rates are often associated with damage to rigid plasma membrane integrity. Cooling and freezing cause a decreasing in the molecular motion of the lipid bilayer plasma membrane, and thus change membrane fluidity (Arav et al., 1996). Actin microfilaments play a central role in the membrane trafficking of many cellís signaling pathways, and are also linked to the plasma membrane as cell cytoskeleton. In this study, we demonstrated that cytochalasin B improved the cryopreservability of immature porcine. The maturation rate of porcine oocytes treated with CB prior to OPS vitrification (MII rate 34.9%) was significantly greater than non-cytochalasin B pretreated controls, similar to the report of Isachenko et al. (1998). Fujihira et al. (2004) found that pretreatment with 7.5 μg/ml CB for 30 min was optimal for porcine oocytes wishing for vitrification, while <5 μg/ml CB demonstrated lower efficiency. Although the mechanism underlying the improved

cryosurvival by cytochalasin B is not clear, depoly-merization of actin microfilament has been postulated to reduce damage during cryopreservation or cell manipulation, possibly by increasing the plasma membrane flexibility (Dobrinsky et al., 2000).

In conclusion, this study demonstrates that immature porcine oocytes can be successfully cryopreserved by means of the success of *in vitro* maturation of vitrified-warmed oocytes, although vitrifying the immature oocytes markedly reduces their developmental competence. Actin depolymerization by cytochalasin B reduces cryoinjury occurring during vitrification and the warming of porcine immature o ocytes. It remains unclear, however, whether these oocytes are essentially capable of fertilization and embryo development.

Acknowledgments

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