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Effects of Partial Removal of Cytoplasmic Lipid on Survival of Vitrified Germinal Vesicle Stage Pig Oocytes

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Abstract. This study was designed to investigate whether the partial removal of cytoplasmic lipid from immature pig oocytes prior to vitrification had any positive effects on subsequent maturation, fertilization and early development. Oocytes at the germinal vesicle stage were partially freed from cumulus cells and centrifuged, and then polarized cytoplasmic lipid was removed by micromanipulation. When cultured for 44–48 h, significantly fewer of the centrifuged oocytes reached metaphase II (M-II) than did the non-centrifuged oocytes (~53% vs ~68%, respectively); however, no further reduction in the M-II rate was observed when centrifuged oocytes were then delipated prior to culture (~47%). To evaluate their sensitivity to the equilibration and vitrification solutions containing ethylene glycol, non-centrifuged, centrifuged, and delipated oocytes were cultured continuously for several minutes in those solutions, then washed and cultured further; no significant differences in the M-II rates (~20–27%) were observed among the three treatment groups. When oocytes were vitrified and then warmed, significantly more delipated oocytes reached M-II in culture (~15%) than did the non-delipated oocytes, whether centrifuged or not (~4% in each group). When delipated, vitrified and matured oocytes were microsurgically injected with frozen-thawed spermatozoa, ~39% were activated and male pronucleus formation was observed in ~40% of activated oocytes; none developed beyond the 4-cell stage. These results show that maturation *in vitro* of vitrified pig oocytes can be promoted by partial removal of cytoplasmic lipid prior to vitrification and that the vitrified oocytes can be fertilized, although the embryonic development obtained in this study was limited.

Key words: Intracytoplasmic sperm injection, Oocyte, Oocyte maturation, Pig, Vitrification

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In both pigs and cattle, embryos for transfer and/or various reproductive technologies can be produced from immature germinal vesicle (GV) stage oocytes, using ovaries obtained from slaughterhouses, after maturation, fertilization and culture *in vitro*. Therefore, if immature oocytes could be effectively cryopreserved, their availability for embryo production, as well as various experimental investigations [1–3], would

be greatly increased. However, cryopreservation of GV stage oocytes is usually more difficult than that of mature oocytes, zygotes or later stage embryos [4–7]. Cryopreservation of immature oocytes has been reported with varying degrees of success in several mammalian species, including mice [2, 8, 9], cattle [3] and humans [10–14]. Although it has been reported that pig GV oocytes do not survive cooling to temperatures below 15 C [15], successful cryopreservation of pig GV oocytes has recently been reported by Isachenko *et al.* [16]. They found

that about 6% of vitrified GV oocytes can survive and mature to metaphase II (M-II) in culture when cryoprotectant saturation and subsequent removal phases were done in a stepwise manner. Under such conditions, they also found that treatment of oocytes with cytochalasin B, a cytoskeletal inhibitor, before vitrification significantly increased the proportion (22%) of oocytes reaching M-II. To date, however, there has been no report of whether vitrified GV pig oocytes, after warming and maturation, can undergo fertilization and subsequent embryonic development.

Since pig oocytes and embryos are extremely sensitive to low temperatures [17, 18], successful cryopreservation has been limited. This problem was overcome partly by Nagashima *et al.* [19, 20] who reported that removal of cytoplasmic lipids from 2- to 4-cell stage pig embryos markedly improved their viability after conventional slow freezing, with live piglets being obtained after transfer of frozen-thawed embryos. Delipation prior to vitrification has also been reported to improve the viability of 2- to 4-cell embryos [21] and morulae/early blastocysts [22]. Although these results indicate that intracellular lipid probably contributes to the pronounced sensitivity of pig embryos to cryopreservation, successful vitrification of non-delipated pig embryos at the morula to hatched blastocyst stages has been reported [23–25]. This suggests that the harmful effects of cytoplasmic lipid on survival of cryopreserved pig oocytes and embryos may depend on their developmental stage.

The present study was designed to examine whether the viability of vitrified GV stage pig oocytes could be improved by delipation, evaluating their maturation in culture, fertilization, and early development *in vitro*. To evaluate fertilization of matured oocytes, we used a technique of intracytoplasmic sperm injection (ICSI), because in a pilot study, we could get no sperm penetration of vitrified oocytes under the routine conditions used for *in vitro* fertilization.

Materials and Methods

Media and solution

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The basic medium used

for collection and vitrification of oocytes was phosphate-buffered saline (PBS; pH 7.4) supplemented with 3 mg/ml BSA (A-4378) (PB1 [26]). For equilibration, ethylene glycol (EG) was diluted to 20% (v/v) in PB1, and this solution was designated EG20. For vitrification, EG was diluted to 40% (v/v) with Ficoll-sucrose (FS) solution; the components of FS solution were 30% (w/v) Ficoll 70 (average molecular weight 70,000; Amersham Biosciences, Uppsala, Sweden) and 0.5 M sucrose in PB1. This vitrification solution was designated EFS40 [27]. The final concentrations of Ficoll 70 and sucrose were 18% (w/v) and 0.3 M, respectively, in EFS40. For dilution, PB1 medium containing 0.5 M sucrose (S-PB1) was prepared. The medium used for maturation of oocytes was BSA-free NCSU 23 [28] supplemented with 0.1 mg/ml cysteine, 5 μ g/ml insulin (I-6634), 10% (v/v) porcine follicular fluid (pFF), 10 IU/ml human chorionic gonadotropin (Gonadotropin; Teikoku-Zoki Co., Tokyo, Japan), 10 IU/ml equine chorionic gonadotropin (Serotropin; Teikoku-Zoki), 75 μ g/ml potassium penicillin G and 50 μ g/ml streptomycin sulphate [29]. Using an 18-gauge needle fixed to a 10-ml disposable syringe, the pFF was withdrawn from superficial follicles (3–6 mm in diameter) in pig ovaries from maturing gilts and then centrifuged at $1,500 \times g$ for 15 min at room temperature (20–25 C) to remove any cells or debris; the supernatant was stored at –20 C until used [30]. The medium used for washing spermatozoa and for ICSI was TL-Hepes-PVA (pH 7.4) composed of 114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO_3 , 0.34 mM NaH_2PO_4 , 0.5 mM MgCl_2 , 2 mM CaCl_2 , 12 mM sorbitol, 10 mM Hepes, 10 mM sodium lactate, 0.2 mM sodium pyruvate, 25 μ g/ml gentamycin, 65 μ g/ml potassium penicillin G and 1 mg/ml polyvinylalcohol (PVA). The medium used for culture of oocytes after ICSI was NCSU 23 supplemented with 4 mg/ml BSA (A-0281; fatty acid free).

Preparation of oocytes

Ovaries from maturing gilts were obtained at a local slaughterhouse and transported to the laboratory within 1–1.5 h in 0.9% (w/v) NaCl solution containing 75 μ g/ml potassium penicillin G and 50 μ g/ml streptomycin sulphate at 37–39 C. Oocytes were aspirated from antral follicles (3–6 mm in diameter) with an 18-gauge needle fixed to a 10-ml disposable syringe; those surrounded by

compact and dense cumulus cell layers were selected in TL-Hepes-PVA. After washing three times with maturation medium, oocytes were partially freed from cumulus cells by repeated passage through a fine pipette in maturation medium supplemented with 0.1% (w/v) hyaluronidase (H-3506). The partially denuded oocytes were washed three times with PBS containing 20% (v/v) fetal bovine serum and 7.5 $\mu\text{g}/\text{ml}$ cytochalasin B, and about 30 oocytes with homogeneous cytoplasm were introduced into 200 μl of the same medium in a 1.5-ml Eppendorf centrifuge tube. The centrifuge tubes were kept at room temperature for about 10 min and then centrifuged at $12,000 \times g$ for 10 min. After centrifugation, oocytes were transferred into 500 μl of TL-Hepes-PVA covered with paraffin oil (No. 261-17; Nacalai Tesque, Kyoto, Japan) in a culture dish (35×10 mm; Falcon No. 1008, Becton Dickinson Labware, Lincoln Park, NJ, USA). The oocyte was held by a holding pipette, with an external diameter of 100–110 μm and an internal opening of 15–20 μm attached to a micro-manipulator (Narishige, Tokyo, Japan) under an inverted microscope; lipid removal was carried out using a beveled suction pipette with an inner diameter of 20–25 μm . Centrifugation causes polarization of cytoplasmic lipid, with a very dense layer closest to the plasma membrane and a less dense layer towards the center of the oocyte (Fig. 1A); delipation involved removal of most of the dense layer of cytoplasmic lipid (Fig. 1B). Non-centrifuged, centrifuged and delipated oocytes were kept in 500 μl of TL-Hepes-PVA covered with paraffin oil in a culture dish until they were used

for maturation *in vitro* or vitrification.

Vitrification of oocytes

Oocytes were vitrified in EFS40 in 0.25 ml plastic straws following the procedure described by Kasai *et al.* [27]. All the procedures were conducted in a room at 25 C. In a straw, a large column of S-PB1 medium (~ 60 mm) and two columns of EFS40 (~ 5 and ~ 12 mm) were aspirated and separated by air (25–30 and ~ 5 mm). Oocytes were first exposed to EG20 for 10 min and then 11–15 oocytes were transferred into the larger column of EFS40. After exposure of oocytes to EFS40 for 1 min, the straw was placed horizontally on a styrofoam boat positioned in liquid nitrogen vapor 1 cm over the liquid surface for at least 3 min in a Dewar vessel (inner diameter, 140 mm; the average cooling rate between 0 and -150 C was ~ 120 C/min), then immersed into liquid nitrogen.

After being stored in liquid nitrogen for at least 1 day, each straw was kept in air for 10 sec and then immersed in water at 25 C. The average warming rates between -196 and -100 C, and -100 C and 0 C were ~ 600 and 1500 C/min, respectively. When the crystallized S-PB1 medium in the straw began to melt (after about 7 sec), the straw was removed from the water and quickly wiped dry; the contents of the straw were expelled into a watch glass by flushing the straw with 0.8 ml of S-PB1 medium. After gently agitating the watch glass to promote mixing of the contents, the oocytes were pipetted into fresh S-PB1 medium. About 5 min after being flushed out, the oocytes were transferred to fresh PB1 medium.

Fig. 1. A pig oocyte at the germinal vesicle (GV) stage before (A) and after (B) partial removal of cytoplasmic lipid polarized by centrifugation at $12,000 \times g$ for 10 min. The GV (arrow) is clearly visible in each figure. Bar=20 μm .

Evaluation of oocyte maturation in vitro

Both non-vitrified and vitrified/warmed oocytes were washed three times with maturation medium; 10–15 oocytes were transferred into a 100- μ l drop of the same medium covered with paraffin oil in a culture dish (35 $\times$ 10 mm) and equilibrated under a humidified atmosphere of 5% CO₂ in air at 39 C for at least 3 h. After culture for 44–48 h under the same atmospheric conditions at 39 C, oocytes were fixed for about 72 h in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid in water, and examined for evidence of nuclear maturation using a phase-contrast microscope at a magnification of $\times$ 200 or $\times$ 400.

ICSI of partially delipated oocytes

Ten to fifteen non-vitrified and 5–6 vitrified oocytes, in which the first polar body was observed after maturation culture, were transferred into 5 μ l of TL-Hepes-PVA covered with paraffin oil in a culture dish lid (50 $\times$ 4 mm; Falcon No. 1006) and kept in a CO₂ incubator (5% CO₂ in air at 39 C) until used for ICSI. Oocytes matured without delipation and vitrification served as the controls. Those oocytes were introduced into $\sim$ 200 μ l maturation medium in a 1.5-ml centrifuge tube, and centrifuged at 7,000 $\times$ g for 10 min to visualize the extruded first polar body.

Frozen-thawed boar semen ($\sim$ 200 μ l) was diluted with 10 ml TL-Hepes-PVA, centrifuged at 600 $\times$ g for 10 min, and the supernatant was removed; pelleted spermatozoa were washed twice with 10 ml TL-Hepes-PVA by centrifugation at 600 $\times$ g for a period of 10 min each. A portion (100 μ l) of the washed sperm suspension was transferred into another centrifuge tube containing about 2 ml TL-Hepes-PVA and $\sim$ 50 μ l of this suspension was again diluted by adding 50 μ l of TL-Hepes-PVA supplemented with 6% (w/v) polyvinylpyrrolidone (PVP-360). A 5- μ l drop of the final sperm suspension was transferred onto the culture dish lid, close to the drop containing the prepared oocytes.

Microinjection of whole spermatozoa into oocytes was performed at 25 C using a piezo-driven pipette that was prepared from borosilicate glass capillary tubes (Sutter Instrument Co. Novato, CA,

USA). The external and internal diameters of the tip of the injection pipette were 10–11 μ m and 8–9 μ m, respectively. A spermatozoon was aspirated into the injection pipette with a minimal amount of medium and the tip of the pipette was brought in contact with the zona pellucida of the oocyte held by a holding pipette with an external diameter of 100–110 μ m and an internal opening of 15–20 μ m. The zona was drilled by applying two to three pulses (intensity 3–5, speed 3). Once the tip had reached the perivitelline space, it was forced onto the oolemma and inserted into the cytoplasm. After a small amount of cytoplasm had been withdrawn to confirm that the tip was actually in cytoplasm, the pipette's contents were expelled into the oocyte; the pipette was then gently withdrawn. The injection of spermatozoa into oocytes was completed within 1 h after preparation of oocytes.

Examination of activation and early development of ICSI oocytes

After ICSI, oocytes were washed three times with culture medium and then 10–15 non-vitrified and 5–6 vitrified oocytes were transferred into 100 μ l of the same medium covered with paraffin oil in a culture dish (35 $\times$ 10 mm; Falcon No. 1008) and cultured in a CO₂ incubator (5% CO₂ in air at 39 C). At 18–20 h after the start of culture, oocytes were fixed and stained as described above; using a phase-contrast microscope at a magnification of $\times$ 200 or $\times$ 400, they were examined for morphological normality, evidence of oocyte activation and transformation of sperm nuclei into male pronuclei. The oocytes with one female pronucleus and the second polar body or with two female pronuclei and only the first polar body were categorized as activated. In another experiment, the oocytes were examined under a dissecting microscope for their development following 48, 72, 144 and 168 h of culture.

Statistical analysis

All proportional data were subjected to an arcsine transformation, and the transformed values were analyzed using one-way ANOVA. When ANOVA revealed a significant effect, the treatments were compared by Fisher's protected LSD least significant difference test.

Results

Maturation in vitro of centrifuged and partially delipated oocytes

As shown in Table 1, significantly fewer ($P < 0.05$) of the centrifuged oocytes had reached metaphase II (M-II) after 44–48 h of culture than had the non-centrifuged control oocytes. However, there was no significant difference between the M-II rates obtained with centrifuged oocytes, with or without delipation after centrifugation; ~50% of these oocytes matured to M-II.

Maturation in vitro of oocytes exposed continuously to equilibration and vitrification solutions after centrifugation and partial delipation

When incubated continuously in equilibration and vitrification solutions, for 10 and 1 min, respectively, washed and then cultured for 44–48 h, some oocytes showed signs of degeneration, including shrinking or blanching of the ooplasm. As shown in Table 2, the proportion of centrifuged oocytes with normal morphology was similar to that in the control group (~65–70%), but significantly fewer ($P < 0.05$) of the centrifuged, delipated oocytes were normal when compared with controls (~56%). However, a similar proportion (~20–27%) of cultured oocytes in all

Table 1. Effect of centrifugation and partial delipation on *in vitro* maturation of partially denuded pig oocytes^a

Oocytes	No. of oocytes cultured	% (mean $\pm$ SEM) of oocytes	
		At the GV stage	Matured to M-II
Control	100	0	68.0 $\pm$ 2.4 ^b
Centrifuged	103	1.0 $\pm$ 0.9	53.4 $\pm$ 2.9 ^c
Delipated	93	7.5 $\pm$ 2.2	47.3 $\pm$ 2.8 ^c

^a Experiments were repeated five times. GV, germinal vesicle; M-II, metaphase II.

^{b, c} Values with different superscripts within column differ significantly ($P < 0.05$).

Table 2. Maturation *in vitro* of partially denuded pig oocytes exposed continuously to equilibration and vitrification solutions after centrifugation and partial delipation^a

Oocytes	No. of oocytes cultured	% (mean $\pm$ SEM) of oocytes		
		Morphologically normal	At the GV stage	Matured to M-II
Control	60	70.0 $\pm$ 3.2 ^b	8.3 $\pm$ 2.3	26.7 $\pm$ 4.4
Centrifuged	65	64.6 $\pm$ 2.2 ^{bc}	9.2 $\pm$ 2.1	21.5 $\pm$ 2.1
Delipated	80	56.2 $\pm$ 2.7 ^c	6.2 $\pm$ 2.5	20.0 $\pm$ 2.8

^a Experiments were repeated four times. GV, germinal vesicle; M-II, metaphase II.

^{b, c} Values with different superscripts within column differ significantly ($P < 0.05$).

Table 3. Maturation *in vitro* of partially denuded pig oocytes vitrified after centrifugation and partial delipation^a

Oocytes	No. of oocytes cultured	% (mean $\pm$ SEM) of oocytes		
		Morphologically normal	At the GV stage	Matured to M-II
Control	80	57.5 $\pm$ 2.1	8.7 $\pm$ 3.4	3.7 $\pm$ 3.5 ^b
Centrifuged	80	52.5 $\pm$ 1.8	11.2 $\pm$ 2.4	3.7 $\pm$ 3.1 ^b
Delipated	80	47.5 $\pm$ 2.0	6.2 $\pm$ 1.7	15.0 $\pm$ 1.9 ^c

^a Experiments were repeated four times. GV, germinal vesicle; M-II, metaphase II.

^{b, c} Values with different superscripts within column differ significantly ($P < 0.05$).

three groups reached M-II.

Maturation in vitro of vitrified oocytes after centrifugation and partial delipation

In this experiment, almost all of the vitrified oocytes were recovered following warming (data not shown) and these were cultured to allow maturation. As shown in Table 3, ~48–58% of oocytes maintained a normal morphology after culture for 44–48 h, with no significant differences among the three treatment groups. However, in terms of oocytes reaching M-II, significantly more ($P<0.05$) of the centrifuged, delipated oocytes reached M-II (~15%; Fig. 2) than did the control and centrifuged oocytes (~4% in each group).

Fig. 2. A partially delipated pig oocyte matured to metaphase II (arrow), with the first polar body (arrow head), 44 h after the start of culture following vitrification; the inset shows a higher magnification of the area within the box. Bar=20 μ m.

Activation and early development of partially delipated oocytes matured after vitrification and injected with spermatozoa

At 18–20 h after sperm injection, ~71–73% of the non-vitrified matured oocytes, whether delipated or not delipated, were morphologically normal (Table 4). In contrast, significantly fewer ($P<0.05$) of the delipated and then vitrified oocytes matured (~42%). With regards to activation and development following ICSI, significantly fewer ($P<0.05$) of the delipated oocytes, whether vitrified or not, activated and had a male pronucleus (Figs. 3A and 3B), compared with untreated controls. Looking at early embryonic development, significantly fewer (~30%; $P<0.05$) of the delipated oocytes underwent early cleavage and even fewer (~16%; $P<0.05$) of the delipated, vitrified oocytes cleaved, compared with the controls (~52%; Table 5). Only ~8% of delipated, vitrified oocytes injected with spermatozoa after maturation developed to the 4-cell stage (Fig. 3C); no development was observed beyond that stage.

Discussion

The aim of the present study was to determine whether removal of cytoplasmic lipid from GV stage pig oocytes enhanced their survival after vitrification. Although it is relatively easy to remove cytoplasmic lipid following centrifugation of pig embryos [19–22], this proved to be more difficult in GV stage oocytes. In a pilot study, we found that the cumulus cells make it very difficult to determine the positions of the GV and polarized cytoplasmic lipid. Therefore, it was necessary to

Table 4. Activation and male pronuclear formation of partially denuded pig oocytes that were partially delipated, matured *in vitro* after vitrification, and injected with frozen-thawed spermatozoa^a

Oocytes	Vitrification	No. of oocytes injected	Morphologically normal	% (mean $\pm$ SEM) of oocytes ^b		
				Total ^c	Activated	
					With enlarged sperm head ^d	With male pronucleus ^d
Control	—	41	70.7 $\pm$ 1.4 ^e	55.2 $\pm$ 3.0 ^e	31.3 $\pm$ 3.6 ^e	68.7 $\pm$ 3.6 ^e
Delipated	—	36	72.7 $\pm$ 3.8 ^e	34.6 $\pm$ 4.6 ^f	55.6 $\pm$ 5.5 ^f	44.4 $\pm$ 5.5 ^f
Delipated	+	31	41.9 $\pm$ 4.0 ^f	38.5 $\pm$ 5.8 ^f	60.0 $\pm$ 14.6 ^f	40.0 $\pm$ 14.6 ^f

^a Experiments were repeated four (non-vitrified) and six (vitrified) times.

^b Oocytes were examined 18–20 h after injection.

^c Percentage of morphologically normal oocytes.

^d Percentage of activated oocytes.

^{e, f} Values with different superscripts within each column differ significantly ($P<0.05$).

Fig. 3. Partially delipated pig oocytes activated with male pronucleus (A) and cleaved to the 4-cell stage (C) 18 and 72 h after sperm injection, respectively. The area within the box in A is shown at higher magnification in B. The oocytes in which the first polar body was observed at 44–45 h of maturation culture after vitrification were injected with frozen-thawed spermatozoa. Two pronuclei (PN) can be seen in A, and the first (PB1) and second (PB2) polar bodies are visible in B. Bar=20 μ m.

Table 5. Development in culture of partially denuded pig oocytes that were partially delipated, matured *in vitro* after vitrification, and injected with frozen-thawed spermatozoa^a

Oocytes	Vitrification	No. of oocytes cultured ^b	% (mean $\pm$ SEM) of oocytes developed			
			≥ 2 -cell (48) ^c	≥ 4 -cell (72) ^c	Morula (144) ^c	Blastocyst (168) ^c
Control	—	56	51.8 $\pm$ 2.4 ^d	39.3 $\pm$ 1.7 ^d	17.8 $\pm$ 1.8 ^d	7.1 $\pm$ 1.6
Delipated	—	37	29.7 $\pm$ 3.5 ^e	18.9 $\pm$ 3.1 ^e	5.4 $\pm$ 2.5 ^e	0
Delipated	+	25	16.0 $\pm$ 3.0 ^f	8.0 $\pm$ 2.8 ^f	0	0

^a Experiments were repeated 4 (non-vitrified) and 5 (vitrified) times.

^b All oocytes injected were cultured.

^c Numbers in parentheses indicate the time after sperm injection.

^{d, e, f} Values with different superscripts within each column differ significantly ($P < 0.05$).

partially remove cumulus cells from centrifuged oocytes to allow adequate visualization of the lipid to be removed. Although a minority of cumulus-free pig oocytes can complete meiotic maturation *in vitro* [31], the retention of cumulus cells appears to be important for oocytes to maintain their ability to mature to M-II [32–35]. Using the same maturation medium as in the present study, Abeydeera *et al.* [29] reported that about 88% of cumulus-intact GV pig oocytes could mature to M-II. In the present study, ~68% of the oocytes maintained their ability to complete maturation, suggesting that partial removal of cumulus cells does not severely affect maturation. However, the proportion of non-vitrified oocytes maturing to M-II was significantly lower if they were centrifuged before maturation; interestingly, delipation following centrifugation did not cause any further significant reduction in the M-II rates. These results suggest that

cytoplasmic lipid is not required by immature oocytes in order for them to mature *in vitro*, at least under the present experimental conditions.

In the present study, EFS40, an EG-based solution, was used for vitrification of oocytes. This was first developed for mouse embryos as a low-toxicity solution [27], and has proven effective for embryos of various mammalian species including the rabbit [36], cattle [37], horse [38], pig [23, 25], sheep [39], human [40] and rat [41]. Isachenko *et al.* [16] have reported that stepwise exposure of cumulus-intact GV pig oocytes to increasing concentrations (5–40%) of EG significantly increased the proportion of oocytes that matured in culture (75–90%), compared with a single-step exposure to 40% EG (40–50%). In a preliminary experiment, however, we found that most delipated oocytes fragmented during stepwise exposure to EG (data not shown). Therefore,

oocytes were just continuously exposed to equilibration (EG20; 20% EG) and vitrification (EFS40; 40% EG) solutions for 10 and 1 min, respectively, before vitrification. Under these conditions, only about 27% of partially denuded GV oocytes matured to M-II in culture. However, similar maturation rates were obtained with centrifuged oocytes, whether delipated or not, indicating that the experimental manipulations did not increase their susceptibility to EG damage.

Cytochalasin B has been used to reversibly depolymerise actin microfilaments in bovine [42, 43] and porcine [25] embryos before or during vitrification to improve their viability after warming cytochalasin B (7.5 $\mu\text{g}/\text{ml}$) treatment for 10–15 min before exposure to EG was reported to increase significantly the proportion of vitrified GV pig oocytes that resumed meiosis (from ~7% up to 22%) [16]. In the present study, partially denuded GV oocytes were kept in a solution containing 7.5 $\mu\text{g}/\text{ml}$ cytochalasin B for 20 min (including the 10 min centrifugation step for centrifuged oocytes) before exposure to EG. Even so, only ~4% of non-centrifuged and centrifuged oocytes matured to M-II after vitrification. Although we did not examine the maturation rate of oocytes not treated with cytochalasin B, the rate obtained in the present study, with cytochalasin B, is lower than that reported by Isachenko *et al.* [16]. The reasons for this are not clear. However, a significantly higher maturation rate (~15%) was obtained when oocytes were delipated prior to vitrification, suggesting that intracellular lipid contributes to the sensitivity of GV oocytes to cryopreservation, as shown in pig [19–22] and cattle [44, 45] embryos.

Despite the low figures for maturation, about 42% of oocytes vitrified after delipation had maintained normal morphology when evaluated 18–20 h after ICSI. Of the morphologically normal oocytes, ~39% were activated and MPN formation was observed in ~40% of the activated oocytes. Although significantly fewer of the delipated, vitrified oocytes were morphologically normal, compared with the non-vitrified oocytes, the activation rate and the incidence of MPN formation were similar in the two groups of oocytes. However, delipation per se was associated with a lower activation rate and a lower incidence of MPN formation, compared with the non-delipated, non-vitrified controls. Thus the fertilizability of matured oocytes is reduced by delipation but not

by vitrification. Delipation also reduced early cleavage of non-vitrified oocytes following ICSI and vitrification following delipation reduced cleavage rates even more, with 8% developing *in vitro* to the 4-cell stage, compared with 39% in the control group, suggesting that both delipation and vitrification contributed to the reduced developmental potential of the manipulated oocytes.

Endogenous lipids play a significant role in cell structure and function, especially in modifying the physical properties and metabolic function of biological membranes [46]. It has been reported that triglycerides are the major component of intracellular lipids in immature pig oocytes [47], and that the triglyceride content of immature pig oocytes significantly decreases during *in vitro* maturation, indicating that it may be used as an energy source [48]. This might account for the reduced incidence of activation and cleavage we obtained with immature oocytes delipated prior to maturation. However, normal piglets have been obtained from 2- to 4-cell stage embryos cryopreserved after delipation [20], and delipated 1-cell bovine embryos can develop to the blastocyst stage *in vitro*, with normal calves being obtained after transfer of the blastocysts [43]. Further study is needed to determine the functional role of cytoplasmic lipid in immature pig oocytes during maturation.

In conclusion, we have demonstrated for the first time that removal of cytoplasmic lipid enhances maturation *in vitro* of vitrified GV pig oocytes. Furthermore, some of those matured oocytes can be successfully activated, forming a male pronucleus and then developing to the 4-cell stage in culture after ICSI with frozen-thawed spermatozoa. However, further experiments are needed to develop more effective methods to support complete development of GV oocytes matured after vitrification.

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