Effects of Meiotic Stages, Cryoprotectants, Cooling and Vitrification on the Cryopreservation of Porcine Oocytes**

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ABSTRACT : Different factors may affect the sensitivity of porcine oocytes during cryopreservation. The effect of two methods (cooling and vitrification), four cryoprotectants [glycerol (GLY), 1, 2-propanediol (PROH), dimethyl sulfoxide (DMSO) or ethylene glycol (EG)] and two vitrification media (1 M sucrose (SUC)+8 M EG; 8 M EG) on the developmental capacity of porcine oocytes at the germinal vesicle (GV) stage or after IVM at the metaphase II (M II) stage were examined. Survival was assessed by FDA staining, maturation and cleavage following IVF and IVC. A toxicity test for different cryoprotectants (GLY, PROH, DMSO, EG) was conducted at room temperature before cooling. GV and M II-oocytes were equilibrated stepwise in 1.5 M cryoprotectant and diluted out in sucrose. The survival rate of GV-oocytes in the GLY group was significantly lower (82%, $p<0.01$) than that of the other group (92 to 95%). The EG group achieved a significantly higher maturation rate (84%, $p<0.05$) but a lower cleavage rate (34%, $p<0.01$) than the DMSO group and the controls. For M II-oocytes, the survival rates for all groups were 95 to 99% and the cleavage rate of the GLY group was lower than the PROH-group (21 vs 43%, $p<0.01$). After cooling to 10°C, the survival rates of GV-oocytes in the cryoprotectant groups were 34 to 51%, however, the maturation rates of these oocytes were low (1%) and none developed after IVF. For M II-oocytes, the EG group showed a significantly higher survival rate than those of the other cryoprotectant groups (40% vs 23-26%, $p<0.05$) and the cleavage rates of PROH, DMSO and EG group reached only 1 to 2%. For a toxicity test of different vitrification media, GV and M II-oocytes were equilibrated stepwise in 100% 8 M EG (group 1) and 1 M SUC + 8 M EG (group 2) or equilibrated in sucrose and then in 8 M EG (SUC+8 M EG, group 3). For GV-oocytes, the survival, maturation and cleavage rates of Group 1 were significantly lower than those in group 2, 3 and control group ($p<0.05$). For M II-oocytes, there were no differences in survival, maturation and cleavage rates between groups. After vitrification, the survival rates of GV and M II-oocytes in group 2 and 3 were similarly low (4-9%) and none of them matured nor cleaved after *in vitro* maturation, fertilization and culture. In conclusion, porcine GV and M II-oocytes do not seem to be damaged by a variety of cryoprotectants tested, but will succumb to a temperature decrease to 10°C or to the process of vitrification, regardless of the cryoprotectant used. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 4 : 485-493)***

Key Words : Cryopreservation, Cryoprotectants, Porcine Oocytes, Cooling, Vitrification

INTRODUCTION

The availability of viable, developmentally competent oocytes has been critical to recent progress in the development of *in vitro* fertilization (IVF), embryo culture and related reproductive technologies such as cloning and genetic engineering in mammal (Parks and Ruffing, 1992). The storage of unfertilized oocytes would generate a readily available source of oocytes for research and allow experiments to be performed at a convenient time (Moor and Crosby, 1985) and could therefore be of practical importance in the establishment of a bank from which particular genetic combinations could be derived (Whittingham, 1977). The relatively short fertile life of mammalian oocytes is a limiting factor in the implementation of many *in vitro* methodologies (Parks and Ruffing, 1992). Up to the present time, unfertilized oocytes have not been effectively cryopreserved.

Frozen-thawed oocytes from several species have been fertilized *in vitro*, but live births after embryo transfer have been reported only for the mouse (Parkening et al., 1976; Whittingham, 1977; Nakagata, 1989, 1993), rabbit (Al-Hasani et al., 1989; Vincent et al., 1989), bovine (Fuku et al., 1992; Otoi et al., 1992, 1993; Suzuki et al., 1996) and human (Chen, 1986; Van Uem et al., 1987), nonetheless, the most suitable maturation stage of oocytes for cryopreservation is still to be determined. Cooling induces abnormalities on a chromosomal level, including disorganization of metaphase plates and multipolar spindles in oocytes cooled at all stages of meiosis from germinal vesicle breakdown (GVBD) to metaphase II (M II) (Suzuki et al., 1996). Oocytes at the GV-stage do not present a microtubular structure of meiotic spindle vulnerable to chilling. Some success has been reported with GV stage oocytes from mouse, rat, rabbit, bovine, pig and human (see references in Parks and Ruffing, 1992). However, several reports indicate that in the bovine (Lim et al., 1992; Otoi et al., 1992; Fuku et al., 1993) and other species (see references

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in Parks and Ruffing, 1992) immature oocytes at the GV-stage are more sensitive to freezing than mature oocytes. In mature oocytes, microtubules appear to be restricted largely to the meiotic spindle with little evidence for foci of pericentriolar material. Cooling also altered the zona pellucida, resulting in decreased sensitivity to chymotrypsin and reduced fertilization rate caused by cortical granule exocytosis occurring prematurely (Suzuki et al., 1996).

It is well known that pig embryos at early stages of development are sensitive to cooling (Wilmut, 1972; Polge et al., 1974; Niemann et al., 1985) and many attemps to cryopreserve porcine embryos have been reported (see references in Nagashima et al., 1994a). However, little information exists on the cooling of porcine oocytes (Didion et al., 1990). The chilling susceptibility demonstrated in immature porcine oocytes has been attributed mainly to destruction of the plasma membrane. Upon cooling, porcine oocytes showed a reduction in membrane potential of the oolemma and various levels of membrane damage (Arav et al., 1996), which was partly due to the high lipid content in porcine oocytes/embryos (Niemann et al., 1985; Nagashima et al., 1994a,b, 1995, 1996). Recently, *in vivo* matured porcine oocytes survived after vitrification and developed beyond the eight-cell stage after removal of lipid and subzonal sperm injection (Nagashima et al., 1996). Live birth of offspring from cryopreserved two to four-cell porcine embryos after removal of lipids has also been reported (Nagashima et al., 1995). Nonetheless, only few informations relating to the suitable maturation stage for cryopreservation, the cooling/freezing protocol and the comparison of the suitablility of different cryoprotectants have been reported.

The aim of the present study was to try to cryopreserve porcine oocytes. The effects of cryopreservation method, maturation status, type of cryoprotectant (permeable or non-permeable) and procedure of dehydration on the capacity for *in vitro* development of frozen-thawed oocytes were investigated.

MATERIALS AND METHODS

Collection of oocytes and follicular fluid

Ovaries were collected from prepuberal gilts slaughtered at the local abattoir. Transportation of the ovaries to the laboratory was carried out in a Dewar flask containing Dulbecco's phosphate buffered saline (PBS) (Serva, Heidelberg, Germany) supplemented with 100 IU Penicillin/ml and 100 µg Streptomycin/ml (Penstrep®, Gibco, Paisley, UK) at 35°C. Within 2 h after slaughter, antral follicles of 2-6 mm diameter were punctured and cumulus-oocyte-complexes (COC) were scraped out into PBS containing 2% fetal calf serum (FCS, Gibco, Paisley, UK) working on a warm plate at 39°C. Oocytes with homogenous cytoplasm surrounded by a compact cumulus mass of at least 3 cell layers were used for the experiments (Huang et al., 2001).

Follicular fluid (pFF) was collected from antral follicles of 2-6 mm diameter of prepuberal gilts 2-4 h after slaughter. The fluid was centrifuged at 1,000 g for 10 min and the supernatant was collected and supplemented with 50 µg/ml of Kanamycin (Sigma, Irvine, UK) and stored at -20°C until use.

In vitro maturation, fertilization and culture

Oocytes were matured for 44-46 h in Medium 199 with Earle's salts (M-199, Sigma, St. Louis, MO, USA), supplemented with 2 Iu hCG/ml (Ekluton®, Intervet, Boxwear, Netherlands), 5 Iu eCG/ml (Intergonan®, Intervet, Boxwear, Netherlands) and 10% pFF. The temperature was maintained at 39°C and the gas atmosphere was 5% CO_2 in air. After maturation, one third of the oocytes were washed in M-199 and denuded mechanically by repeated pipetting. The oocytes were then fixed in acetic alcohol (acetic acid: alcohol=1:3, v/v) for 24-48 h and stained with 1% (w/v) lacmoid in 45% (v/v) acetic acid. Oocytes that had developed to the second metaphase stage or beyond were considered mature.

After *in vitro* maturation or exposure to different cryoprotectants, the oocytes were washed three times by placing them into drops of fertilization medium (modified M-199+2.01 mM caffeine at pH 7.4) (Cheng, 1985). Thirty oocytes were transferred to 35 mm plastic petri dishes (Nunc, Roskilde, Denmark) containing 2 mL fertilization medium. An amount of pre-incubated sperm suspension was added to give a final concentration of 1×10^5 to 1×10^6 /ml at insemination (Cheng, 1985). After coincubation with spermatozoa for 6 h at 39°C in an atmosphere of 5% CO_2 in air, the oocytes were removed from the dishes, washed three times by placing them into drops of mBMOC-2 (Cheng, 1985) to remove excess spermatozoa and transferred to fresh mBMOC-2+10% FCS+0.4% BSA for 96 h at 39°C in an atmosphere of 5% CO_2 in air (Huang et al., 2001). The medium was renewed every 24 h.

All media were filtered through 0.2 µm syringe filters (Sartorius, Goettingen, Germany) just before use.

Fluorescence staining

FDA-staining : As a survival assay after the toxicity-test, cooling and vitrification oocytes were stained with a final concentration of 5 µg FDA mL^{-1} (3'6'-Fluorescein-Diacetate; Sigma, St. Louis, MO, USA) (Didion et al., 1990). The oocytes were placed in a 75 µl droplet of FDA working solution for 3 min, rinsed twice in 75 µl of PBS, transferred to 20 µl of PBS on a microscope slide and examined using a fluorescence microscope (Axioskop, Carl Zeiss, Goettingen, Germany). The classifications of oocytes after FDA staining were 1) viable oocyte and viable cumulus cells (VOC), 2) viable oocyte and nonviable

cumulus cells (VO), 3) nonviable oocyte and viable cumulus cells (VC) and 4) nonviable oocyte and nonviable cumulus cells (NV). Only oocytes with fluorescing ooplasm and cumulus cells (VOC) were considered survivors (figure 1).

Hoechst 33342-staining : The staining with Hoechst 33342 (bisBenzimide, Sigma, Steinheim, Germany) was conducted to evaluate the nuclei of oocytes after IVFC. The method of preparation and staining with Hoechst 33342 has been described elsewhere (Pursel et al., 1985). The Hoechst 33342 stock solution was prepared by dissolving 1 mg/ml of Hoechst 33342 in distilled water.

Cooling and vitrification

Oocytes, either immediately after collection (GV-stage) or after 44-46 h of *in vitro* maturation to the M II stage, were pooled and underwent cooling and vitrification treatments as indicated in figure 2. They were either exposed to 1.5 M glycerol (GLY) (Merck, Darmstadt, Germany), 1.2-propanediol (PROH) (Merck), dimethyl-sulphoxide (DMSO) (Merck) or ethylene glycol (EG) (Sigma, St. Louis, MO, USA) and then cooled to 10 °C or exposed to 8 M EG and 1 M sucrose (SUC) (Serva, Heidelberg, Germany)+8 M EG before vitrification. Before cooling or vitrification, a toxicity test was conducted to evaluate the effect of different cryoprotectants on the viability of GV and M II-oocytes. The maturation rate of the M II-oocytes was evaluated from one third of the oocytes before the above treatments. The basic medium for cooling and vitrification was PBS supplemented with 15% FCS. All manipulations were performed in 100 mm plastic petri dishes (Greiner, Solingen, Germany) at room temperature. The experiments were repeated 3 to 6 times.

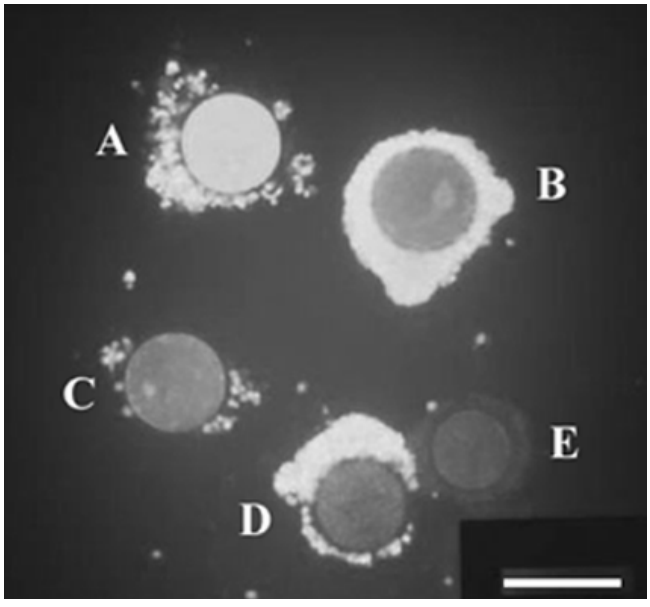

Figure 1. FDA staining after cryopreservation of porcine oocytes

A: both oocyte and cumulus cells are viable
B, C, D: oocyte is nonviable, cumulus cells are viable
E: both oocyte and cumulus cells are nonviable (bar=200 µm)

Cooling : For the toxicity test, oocytes at the GV and M II-stage were equilibrated in 1.5 M GLY, PROH, DMSO or EG in three steps (0.5 M, 1.0 M and 1.5 M for, respectively, 5, 5 and 10 min). The respective cryoprotectant was diluted out in four steps by serial transfer of oocytes into 0.3 M SUC containing 0.5 M cryoprotectant, 0.3 M SUC, 0.15 M SUC and then into the IVM/IVF-medium for 5 min at a time. A control group was cultured in PBS supplemented with 15% FCS without any cryoprotectant (untreated oocytes). Immediately after dilution, one third of either GV or M II-oocytes was stained with FDA to determine their respective survival rate. The other two third of the M II-oocytes were fertilized and cultured (IVFC), while the other two thirds of the GV-oocytes were in vitro-matured before undergoing the similar treatment (IVMFC). Maturation and cleavage rates were determined after the treatments (figure 2).

For cooling to 10°C, GV and M II-oocytes were equilibrated in either of the four cryoprotectants as described above. Ten to 15 oocytes were then loaded into the central portion of a 0.25 mL straw (Minitueb, Landshut, Germany) containing 30 µL of 1.5 M cryoprotectant. Both ends of the straw were filled with 0.3 M sucrose containing 1.5 M cryoprotectant, separated from the medium containing the oocytes by air bubbles. The straws were then sealed with plastic plugging rods and transferred to a cryostat (F3-Q, Haake, Karlsruhe, Germany) at room temperature. They were then cooled to 10°C at 1°C/min. After 10 min at 10°C and rewarming to room temperature by immersing straws in 20°C water for 15 sec, the oocytes were expelled into petri dishes. Dilution, followed by washing, testing for survival and culture were performed as described above.

Vitrification : For the vitrification experiments, ethylene glycol and sucrose were chosen as cryoprotectants. To test for the toxicity of these substances, oocytes at the GV and M II stages were equilibrated for 30 sec in 25%, 50%, 75% and then for 1 min in 100% 8 M EG (group 1) and 1 M SUC + 8 M EG (group 2). Another group was equilibrated in 0.3 M, 0.5 M and 1.0 M sucrose for 1 min and then transferred in 8 M EG for 1 min (SUC+8 M EG; Group 3). A control group was cultured in PBS supplemented with 15% FCS without cryoprotectant (untreated oocytes). Oocytes were then diluted in 1 M sucrose for 10 min and in IVM/IVF-medium for another 5 min. After dilution, one third of the oocytes was stained with FDA to determine the

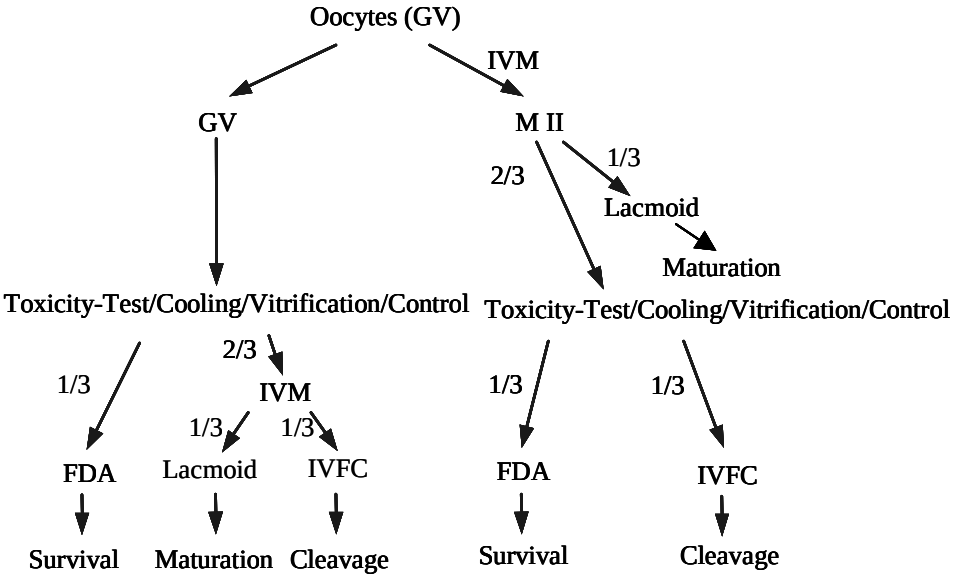

Figure 2. Schematic presentation of the general experimental design

survival status. The other two thirds of GV-oocytes and one third of M II-oocytes underwent a maturation treatment or were fertilized and cultured as presented in figure 2.

Two solutions were selected for vitrification. These were the ones referred to previously as "Group 2" and "Group 3". After equilibration, as described above, oocytes at GV- and M II-stages were loaded into straws and both ends of the straws were filled with 1 M sucrose. Straws were immersed in liquid nitrogen in two steps: the column containing 1 M sucrose was dipped slowly to prevent bursting of the straws and the column containing oocytes and the vitrification solution was dipped rapidly. After 2 to 4 weeks of storage, the straws were exposed to air at room temperature for 5 sec before being immersed in 20°C water for 15 sec. The oocytes in the vitrification solution were expelled into petri dishes and transferred into 1 M sucrose for 10 min and IVM/IVF-medium for 5 min. Survival assay and culture were conducted as described before.

Statistical analysis

The statistical significance of differences between groups and between maturation stages was calculated according to Chi-square (χ^2-test) analysis. A probability of $p<0.05$ was considered to be statistically significant.

RESULTS

Cooling

The purpose of this investigation was to study the effect of an exposure of porcine GV and M II-oocytes to a variety of cryoprotectants at room temperature and while cooling them to 10°C.

The effect of cryoprotectants at room temperature

The effect of an exposure of GV and M II-oocytes to GLY, PROH, DMSO and EG on survival, maturation and cleavage is shown in table 1. The survival of GV-oocytes after exposure to GLY was lower than after exposure to either the other cryoprotectants and the control group (82 vs 92 to 95%, $p<0.01$). Differences among the other groups were not significant. The maturation rate was higher in the EG group than in the PROH and DMSO groups (84 vs 74 and 74%, $p<0.05$), but not significant when compared with the GLY and the control groups (75 and 79%). After IVF, the cleavage rates were higher in the DMSO and control groups, respectively, than in the EG group (44 and 48 vs 34%, $p<0.01$). However, differences among these three groups and the GLY and PROH groups were not significant.

Maturation rates of M II-oocytes prior to exposure to cryoprotectants ranged from 74 to 85%. After exposure to GLY, survival was higher for M II-oocytes than for GV-oocytes (95 vs 82%, $p<0.01$). The cleavage rate of M II-oocytes in the PROH group was better than in the GLY group (43 vs 21%, $p<0.01$). The other differences were not significant. After exposure to GLY, cleavage rate of M II-oocytes was lower than in GV-oocytes (21 vs 39%, $p<0.01$). A similar trend was observed in the control group (32 vs 48%, $p<0.01$).

According to these results, porcine oocytes at the GV- and M II-stages will survive in a short-time exposure to either cryoprotectant examined. Survival, maturation and cleavage rates were not different from the control group.

Cooling to 10°C

All oocytes were recovered after cooling. Table 2 shows

Table 1. The effect of the cryoprotectants glycerol (GLY), 1,2-propanediol (PROH), dimethyl sulfoxide (DMSO) or ethylene glycol (EG) on the developmental capacity of porcine oocytes at germinal vesicle (GV) or metaphase II (M II) stages after exposure at room temperature

Stage	Variable	GLY	PROH	DMSO	EG	Control
GV	No. of oocytes	628	560	554	546	725
	Survival (%)*	$82^{a\,A}$	94^{b}	95^{b}	92^{b}	94^{b}
	Maturation (%)*	75^{cd}	74^{c}	74^{cC}	84^{d}	79^{cd}
	Cleavage (%)*	39^{abA}	38^{ab}	44^{a}	34^{b}	48^{aA}
M II	No. of oocytes	240	224	228	233	245
	Maturation (%)*	80	83	85^{D}	82	82
	Survival (%)*	95^{B}	96	96	99	99
	Cleavage (%)*	21^{aB}	43^{b}	34^{ab}	32^{ab}	32^{abB}

^{a,b} values in the same row with different superscripts differ ($p<0.01$).
^{c,d} values in the same row with different superscripts differ ($p<0.05$).
^{A,B} corresponding values in the same column with different superscripts differ ($p<0.01$).
^{C,D} corresponding values in the same column with different superscripts differ ($p<0.05$).
* Survival (%)=viable oocyte and cumulus cells/total oocytes×100.
Maturation (%)=M II-oocytes/total oocytes×100.
Cleavage (%)=cleaved oocytes/ total oocytes×100.

Table 2. The effect of the cryoprotectants glycerol (GLY), 1,2-propanediol (PROH), dimethyl sulfoxide (DMSO) or ethylene glycol (EG) on the developmental capacity of porcine oocytes at germinal vesicle (GV) or metaphase II (M II) after cooling to 10°C

Stage	Variable	GLY	PROH	DMSO	EG	Control
GV	No. of oocytes	266	250	264	249	307
	Survival (%)*	34^{a}	51^{bA}	48^{bA}	40^{ab}	87^{cC}
	Maturation (%)*	0^{a}	1^{a}	1^{a}	1^{a}	80^{b}
	Cleavage (%)*	0	0	0	0	37
M II	No. of oocytes	278	272	277	288	308
	Maturation (%)*	79	88	84	87	86
	Survival (%)*	23^{a}	26^{aB}	26^{aB}	40^{b}	98^{cD}
	Cleavage (%)*	0^{a}	1^{a}	2^{a}	2^{a}	42^{b}

^{a,b} Values in the same row with different superscripts differ ($p<0.05$).
$^{c\ vs\ a\ and\ b}$ Values in the same row with different superscripts differ ($p<0.001$).
^{A,B} corresponding values in the same column with different superscripts differ ($p<0.001$).
^{C,D} corresponding values in the same column with different superscripts differ ($p<0.01$).
* see foot-note to table 1.

survival, maturation and cleavage rates after cooling to 10°C for 10 min. The survival rate of GV-oocytes was lower in the GLY group than in either PROH, DMSO or control groups (34 vs 51, 48 and 87%, $p<0.05$). There were no differences among PROH, DMSO and EG groups. Only 1% of the oocytes in the PROH, DMSO and EG groups reached the M II stage, and none showed cleavage after IVF. In the control group, however, 37% of cleavage rate was obtained. The maturation rates of the M II-oocytes ranged from 79 to 88%. The survival rate in the EG group was higher than in the GLY, PROH and DMSO groups (40 vs 23 to 26%, $p<0.05$). Cleavage rates after IVF of oocytes exposed to cryoprotectants were between 0 and 2%. After exposure to cryoprotectants, the average of survival rate of GV-oocytes was higher than M II-oocytes ($p<0.01$). In the control group, however, the survival rate of MII-oocytes was higher than GV-oocytes (98% vs 87%, $p<0.01$).

Vitrification

The purpose of this investigation was trying to vitrify porcine oocytes. As a first step we studied the effect of different vitrification media on the developmental capacity of porcine GV and M II-oocytes.

The effect of different vitrification media

As shown in table 3, survival, maturation and cleavage rates of GV-oocytes exposed to 8 M EG (group 1) were lower than for any of the other groups ($p<0.05$). The control group had the highest survival and maturation rate ($p<0.05$), yet its cleavage rate was no different from groups 2 and 3.

Table 3. The effect of various vitrification solutions (8 M EG, 1 M SUC+8 M EG and SUC-8 M EG) on the developmental capacity of porcine oocytes at germinal vesicle (GV) or metaphase II (M II) stages after exposure at room temperature

Stage	Variable	Group 1 (8 M EG)	Group 2 (1 M SUC+8 M EG)	Group 3 (SUC-8 M EG)	Control
GV	No. of oocytes	316	329	334	376
	Survival (%)*	52^{aA}	94^{b}	89^{b}	99^{c}
	Maturation (%)*	51^{aA}	74^{b}	76^{b}	89^{c}
	Cleavage (%)*	16^{a}	30^{b}	29^{b}	33^{b}
M II	No. of oocytes	321	328	326	347
	Maturation (%)*	79^{B}	84	80	83
	Survival (%)*	84^{aB}	87^{a}	88^{a}	95^{b}
	Cleavage (%)*	23	28	31	25

^{a,b} values in the same row with different superscripts differ ($p<0.05$).
$^{c\ vs\ a\ and\ b}$ values in the same row with different superscripts differ ($p<0.001$).
^{A,B} values in the same row with different superscripts differ ($p<0.01$).
* see foot-note to table 1.

In M II-oocytes, survival rates after exposure to cryoprotectants were slightly lower than in the controls (84 to 88% vs 95%, $p<0.05$), with no significant differences among vitrification media. Maturation rates were almost identical, and so were the cleavage rates.

The only noteworthy differences ($p<0.01$) between GV and M II-oocytes were observed in group 1 (8 M EG). The results of group 1 were not as good as those of the other groups. This group was, therefore, omitted from the actual vitrification experiment.

Freezing

The proportion of oocytes recovered after vitrification and thawing was 82% (90% for GV-oocytes and 73.5% for M II-oocytes). Ten percent of the straws exploded during thawing; most of them lost their cotton plug, only few cracked. Post-vitrification survival of GV-oocytes was 9% for both groups. For M II-oocytes, they were, correspondingly, 4 and 9%. None of the oocytes matured or developed after IVM or IVF (table 4), indicating that vitrification caused severe damage to the oocytes.

Table 4. The effect of various vitrification solutions (1 M SUC+8 M EG and SUC-8 M EG) on the developmental capacity of porcine oocytes at germinal vesicle (GV) or metaphase II (M II) stages after vitrification

Stage	Variable	Group 2 (1 M SUC + 8 M EG)	Group 3 (SUC-8 M EG)
GV	No. of oocytes	504	548
	Survival (%)*	9	9
	Maturation (%)*	0	0
	Cleavage (%)*	0	0
M II	No. of oocytes	320	342
	Maturation (%)*	84	82
	Survival (%)*	4	9
	Cleavage (%)*	0	0

* See foot-note to table 1.

DISCUSSION

The first part of this study was devoted to attemps to establish a technique for cooling immature (GV-stage) or *in vitro* matured (M II-stage) porcine oocytes to 10°C. To this avail the following cryoprotectants were tested: GLY, PROH, DMSO and EG. The second part of the paper describes attemps to vitrify porcine GV and M II-oocytes in different vitrification media.

Our data indicate that porcine oocytes could tolerate a variety of cryoprotectants. GV-oocytes exposed to glycerol were slightly inferior compared with oocytes exposed to PROH, DMSO and EG. This became more evident and applied to M II-oocytes as well after cooling to 10°C. Albeit, only 1% of the GV-oocytes showed signs of maturation and none cleaved. The fact that immature oocytes are less permeable than the mature ones and glycerol is less permeant could account for this result (Le Gal and Massip, 1999). Consistent with our results, Miyamoto et al. (1988) used 1.5 M GLY as protectant when cooling porcine GV-oocytes to 10°C and found that none continued to mature *in vitro*. When Didion et al. (1990) cooled porcine GV-oocytes with 1.5 M GLY directly to 15, 10 and 0°C they observed between 77 and 82% morphological intactness, yet none had survived judged by FDA-staining, indicating that direct cooling could be more damaging to the oocytes. Another study (Graves et al., 1995) showed that mature (M II) porcine oocytes could be cooled to at least 4°C and immature (GV) oocytes to 18°C without a decrease in viability after slow or fast cooling/warming judged by fluorescein staining, whereas a decrease in viability is evident when immature oocytes are cooled to 4°C. Our results, however, indicate that a temperature decrease to 10°C is detrimental to the viability of both GV and M II-oocytes after slow cooling and fast warming judged not only by fluorescein staining but also by *in vitro* maturation/fertilization rates.

The microscopic appearance of oocytes exposed to 10°C suggests degeneration. They assume an irregular shape with inhomogenous cytoplasm and a yellow or brownish tint. The detrimental effect was discernible immediately after cooling but became more distinct after the subsequent maturation period in case of the GV-oocytes, and after *in vitro* fertilization in case of the M II-oocytes (unpublished data). Similar observations have been reported in pigs (Didion et al., 1990; Rubinsky et al., 1992; Chen et al., 1995; Wu and Lee, 1996; Gustafsson et al., 2001), and cattle (Schellander et al., 1994; Martino et al., 1996). The cumulus cells surrounding the oocytes did not undergo comparable degenerative changes. They had a survival rate over 90% (data not shown). There are probably differences in plasma membrane lipid properties between somatic (cumulus) and germ (oocyte) cells that would account for this differential sensitivity to cooling as suggested by Didion et al. (1990).

The 10 min holding time at 10°C in our experiment should also be considered. By way of the explanation of cooling injury, Arav et al. (1996) suggest that, in bovine GV-oocytes, a phase transition of the membrane lipids occurs at temperatures between 13 and 20°C and its plasma membrane becomes more sensitive to chilling than in M II-oocytes. They surmise that holding of immature oocytes at the phase transition temperature is more damaging to their membranes than exposure to lower temperatures. For M II-oocytes, the phase transition temperature centered around 10°C and their microtubules and microfilaments could be damaged, which may be related to polyspermic fertilization (Arav et al., 1996). The low maturation and fertilization rate in our results could result from the damages described before.

A protective effect of antifreeze proteins stabilizing plasma membranes and elevating maturation rates of porcine GV-oocytes and the survival of porcine embryos has been described (Rubinsky et al., 1992; Arav et al., 1994). Twenty-nine percent (14/49) of the frozen-thawed *in vivo* matured oocytes collected from local native minipigs cryopreserved with antifreeze protein type III developed to the morula stage after *in vitro* fertilization (Chen et al., 1995). However, whether *in vivo* matured porcine oocytes or oocytes from special strains are suitable for cryopreservation and the protective mechanism of antifreeze proteins is still unclear.

Meiotic spindle, cortical granules and cytoskeleton of mature oocytes are thought to be susceptible to damage during cooling and exposure to cryoprotectants (Rall, 1992; Leibo et al., 1996). Many cryoprotectants bring about depolymerization of cytoskeletal components prior to cooling and may be toxic to cells, and the cytoskeleton may be affected during vitrification. In addition, microfilament depolymerization through the addition of stabilizer (cytochalasin B) prior to cryopreservation improves the development of pig blastocysts (Dobrinsky et al., 1997). Nonetheless, the effect of cytochalasin B on the cryopreservation of porcine oocytes still to be determined.

The lower survival rate of GV-oocytes after exposure to 8 M EG vitrification medium could relate to differences in permeability of immature and mature oocytes, and renders the immature oocyte susceptible to osmotic damage upon addition of a cryoprotectant (Ruffing et al., 1993; Le Gal et al., 1994; Le Gal and Massip, 1999). In case of vitrification, our results showed that only 4 to 9% of porcine GV and M II-oocytes survived and none matured or cleaved. In contrast to cooling, the survival rates of cumulus cells also decreased to lower than 30% (data not shown). These results may be related either by a direct toxic effect of the cryoprotectant itself or to osmotic effects (Rall, 1987; Hochi et al., 1994; Martino et al., 1996; Le Gal and Massip, 1999). Embryos thawed after vitrification had the same microscopic appearence of degeneration described for cooled embryos (unpublished data). In agreement with Hochi et al. (1994), we found that it was easier to mechanically remove cumulus cells from GV- and M II-oocytes after freezing than beforehand. Presumably gap junctions between cumulus cells and oocytes are damaged by the processes of freezing and thawing as reported for the equine (Hochi et al., 1994) or spontaneous dissociation after cell death. In contrast to our results, Dobrinsky et al. (1995) indicated that immature porcine oocytes can survive (33%) after vitrification and remain viable throughout maturation (10%), but their viability is compromised after exposure to a cryoprotectant prior to cryopreservation. In contrast to immature oocytes, the viability of *in vitro* matured oocytes is still compromised even after exposure to liquid nitrogen. Another study (Wu and Lee, 1996) showed that 59% of the GV-oocytes survived vitrification, however, only 12% of the survived oocytes had a homogenous distribution of mitochondria throughout the cytoplasm after fluorescein staining, which indicated that most of the oocytes were cryoinjuried during vitrification.

Not only the toxicity of cryoprotectants, osmotic effect and cryoinjury but also the high lipid content in porcine oocytes and embryos are thought to be responsible for the high sensitivity to low temperature (Niemann et al., 1985; Nagashima et al., 1994a,b). The birth of live piglets from delipated and cryopreserved 2-4-cell porcine embryos (Nagashima et al., 1995) and the development of *in vivo* matured oocytes to the morula stage after delipation, vitrification and subzona sperm injection (Nagashima et al., 1996) confirm this hypothesis. The low number of cleaved embryos after cooling and vitrification in this experiment rendered embryo transfer impossible. At least four good quality embryos are required to induce and maintain pregnancy (Polge et al., 1966).

In conclusion, porcine GV und M II-oocytes do not seem to be damaged by a range of cryoprotectants tested, but will succumb to a temperature decrease to 10°C or to the process of vitrification, regardless of the cryoprotectant used. Failure to achieve any post-cooling or post-freezing survival in either GV or M II oocytes in this elaborate investigation confirms the generally held supposition that preservation of porcine oocytes demands an altogether different approach.

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