Developmental potential and ultrastructural injuries of metaphase II (MII) mouse oocytes after slow freezing or vitrification

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Purpose: The purpose of this study was to determine the developmental ability and ultrastructure of MII mouse oocytes after cryopreservation by slow freezing or vitrification.

Methods: Ovulated MII mouse oocytes were allocated to slow frozen, vitrified and control groups. Oocytes in the slow frozen and vitrified groups were cryopreserved using 1,2 propanediol (PROH) and ethylene glycol (EG) respectively as cryoprotectants. After thawing, the surviving MII oocytes in both cryopreserved groups and the control group were inseminated and their developmental ability was compared. The ultrastructure of MII oocytes in both cryopreserved groups was assessed immediately after thawing and 10 h post insemination at the pronuclear stage, and compared with that of the control group.

Results: The survival rates were nearly identical in both cryopreserved groups. The fertilization rates were also identical and comparable to that of the control group. The further development of vitrified oocytes was similar to that of the control oocytes, whereas it was severely limited in the slow-frozen oocytes. In the slow-frozen MII oocytes, the intermediate filaments were destroyed and the oolemma and microvilli were also modified. At the pronuclear stage deterioration of mitochondria and the presence of numerous vacuoles were also observed within the ooplasm. In the vitrified MII oocytes, the intermediate filaments were the only structures affected and these cytoskeletal elements were reorganized at the pronuclear stage.

Conclusions: Vitrification results in less ultrastructural damage and better post fertilization development of MII mouse oocytes than slow freezing.

KEY WORDS: Mouse oocytes; slow freezing; ultrastructural injuries; vitrification.

INTRODUCTION

The earlier studies on oocyte cryopreservation were carried out by slow freezing methods using different cryoprotective agents such as dimethyl sulphoxide (DMSO) and 1,2 propanediol (PROH) (1–3). PROH has been reported to be useful in the

cryopreservation of mammalian embryos (4), but it seems to be less effective with mammalian oocytes. Murayama *et al.* (5) using PROH, has reported less than 20% of cryopreserved mouse oocytes survived after thawing, whilst Joly *et al.* (6) reported that 1.5 M PROH caused mouse oocytes to form cytoplasmic blebs.

Imoedembe and Sique (7) also reported that PROH could be used in human oocyte cryopreservation but the survival rate after thawing was only 44%. Cryopreservation of oocytes has also been carried out by vitrification using different cryoprotective agents such as the vitrification solution 1 (VS 1; a

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mixture of PROH, DMSO, acetamide and polyethylene glycol) and DMSO (8–11). Several investigators have also reported that ethylene glycol (EG) may be a suitable cryoprotective agent for the vitrification of mammalian oocytes (12–14).

However, in spite of all of these efforts, the reasons for the low success rate of oocyte cryopreservation are not fully understood. It may be due to the change in nature of the zona pellucida or zona hardening by premature release of cortical granules (15–17), loss of the plasma membrane selective permeability (18), or extensive disorganization of ooplasm and damage to the spindle apparatus by disorganization of microtubules and microfilaments during freezing and thawing (6,19,20). Carroll *et al.* (21) observed that the freezing–thawing procedure induced changes in the zona pellucida of oocytes. This hardening is responsible for a decrease in the fertilization rates. Sathananthan *et al.* (22) showed cracks in the zona pellucida, disruption of the plasma lemma and extensive disorganization of ooplasm after freezing of human oocytes using a routine slow freezing method. Zhao *et al.* (23) showed similar results after cryopreservation of bovine oocytes in a solution containing propandiol as cryoprotectant.

Sathananthan *et al.* (24), in an ultrastructural study, showed nuclear abnormalities in fertilized eggs at the pronuclear (PN) stage after vitrification of human and mouse oocytes. Also their results indicated that meiotic spindles were sensitive to cooling.

Hochi *et al.* (25) showed that vitrification induced some ultrastructural changes such as swelling of mitochondria together with reduced matrix density, disorganization of junctions between oocytes and cumulus cells, and also the presence of vacuoles in the periphery of the ooplasm. They concluded that reduction in the viability of vitrified oocytes might be related to morphological changes. They showed no changes in the zona pellucida or the lipid droplets. On the other hand Fuku *et al.* (26) suggested that the premature release of cortical granules and the alteration of the zona pellucida are a possible cause for the low viability of in vitro matured (IVM) cattle oocytes after vitrification. They also showed that vitrification induced ultrastructural modifications in microvilli and mitochondria, and vesicle formation in the ooplasm of germinal vesicle (GV) cattle oocytes (26).

The purpose of this study was to investigate and compare the ultrastructural injuries of MII mouse oocytes from a single mouse strain (NMRI), after routine slow freezing (using PROH) and vitrification

(using an EG-based solution). The reversibility of these changes and developmental capacity of these oocytes from fertilization to hatching blastocyst stage was also assessed.

MATERIALS AND METHODS

Oocytes

Six to ten-week old female NMRI mice were induced to superovulate with an injection of 10 IU human menopausal gonadotropin (hMG; Serono) followed by 10 IU human chorionic gonadotropin (hCG; Serono), 48 h later. Mice were sacrificed 12–14 h post hCG injection by cervical dislocation and oocytes with cumulus cell mass were flushed from the oviducts with T6 medium. The cumulus cells were then dispersed by phosphate buffered saline containing 1% hyaluronidase (Sigma). Morphologically normal MII oocytes were washed and pooled in fresh T6 medium supplemented with 5 mg/ml bovine serum albumin (BSA; Sigma). Seven hundred and fifty-seven MII mouse oocytes were randomly allocated to non-frozen control ($n = 260$), slow frozen ($n = 294$) and vitrified ($n = 203$) groups.

Slow-Frozen Thawed Procedure

Oocytes from the slow-frozen group were frozen using the slow freezing method reported elsewhere (27). Briefly, oocytes were exposed to 1.5 M PROH in phosphate buffered saline supplemented with 5 mg/ml BSA for 15 min, then transferred to 1.5 M PROH and 0.1 M sucrose in phosphate buffered saline supplemented with 5 mg/ml BSA for 15 min. Groups of 15–20 oocytes were loaded into 0.25 ml straws (Stromberg, Germany CTE 880-0.2-EC) which were sealed and placed into a programmed freezer (Stromberg, Germany CTE 880 cryo-Technic). The temperature was gradually decreased to -7°C ($3^{\circ}\text{C}/\text{min}$) at which point seeding of the straws was induced. After 5 min, the temperature was lowered to -30°C at the rate of $0.3^{\circ}\text{C}/\text{min}$. The straws were held at this temperature for 30 min. The oocytes were then rapidly cooled to -196°C and stored in liquid nitrogen. After 2 weeks straws were thawed by transfer from liquid nitrogen to room temperature for 10 s, followed by shaking in a 25°C water bath for 20 s. Cryoprotectant was removed by passing the oocytes through decreasing of PROH (1, 0.5, 0.25, and 0 M) in 0.2 M sucrose in phosphate buffered saline supplemented with 5 mg/ml BSA (5 min for each step).

Finally, the oocytes were washed twice in phosphate buffered saline, scored for survival and transferred to a drop of T6 medium containing 5 mg/ml BSA.

Vitrification and Thawing Procedures

Oocytes of the vitrified group were frozen using the vitrification method reported elsewhere (28). Briefly, the vitrification solution (EFS10) was prepared using 30% (W/V) ficoll 70 (average molecular weight 70000), 0.5 M sucrose, 10.7% (W/V) acetamide and 10% (V/V) ethylene glycol in phosphate buffered saline supplemented with 4 mg/ml BSA. The oocytes were transferred to EFS10 at room temperature and held for 2 min. Groups of 15–20 oocytes were loaded into 0.5 ml straws (A-201-Instruments de médecine vétérinaire, France) as described by Kasai *et al.* (29). The straws were sealed and plunged directly into liquid nitrogen.

After 2 weeks straws were thawed by transfer from liquid nitrogen to room temperature for 20 s, followed by shaking in a 25°C water bath for 25 s. The contents of each straw were expelled into 0.8 ml of 0.5 M sucrose in phosphate buffered for 5 min. The oocytes were washed twice in phosphate buffered saline, scored for survival and transferred to a drop of T6 medium containing 5 mg/ml BSA.

In Vitro Fertilization Procedure

Spermatozoa were extracted from the cauda epididymis of NMRI mice and capacitated for 1.5 h in T6 medium containing 5 mg/ml BSA.

The surviving MII oocytes from both experimental groups and the control non-frozen oocytes were transferred to T6 medium containing 16 mg/ml BSA, inseminated with capacitated sperm and incubated in a humidified 37°C incubator in an atmosphere of 5% CO₂ in air. The culture medium was renewed after 4–6 h.

Assessment of Development and Data Analysis

Experiments were repeated eight times. Fertilization and further development of oocytes was recorded from 10 to 120 h post insemination. Data were analyzed by χ^2 test.

Electron Microscopic Procedure

The ultrastructure of survived MII oocytes from both experimental groups immediately after thawing and at the pronuclear stage was compared with that of non-frozen control oocytes. At least five oocytes from each group were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH: 7.4) for 1 h at 4°C, and washed in the same buffer for 10 min. The oocytes were then post-fixed in 1% osmium tetroxide, washed in phosphate buffer and dehydrated in ascending concentrations of ethanol followed by propylene oxide. The oocytes were embedded in Araldite resin (Merck, Germany), sectioned at a thickness of 50 nm, stained with alcoholic uranyl acetate (7 min) and aqueous lead citrate (13 min) and examined with a TEM-900 electron microscope (Zeiss, Germany).

RESULTS

Survival and Developmental Potential of Cryopreserved Mouse MII Oocytes

The rates of survival of oocytes and their subsequent development are shown in Table I. There was 85% post thaw survival of the slow-frozen oocytes compared to 80% survival of the vitrified oocytes. The rates of fertilization in the control, slow frozen and vitrified oocytes were 92.6, 91.2 and 91.4%, respectively. Twenty four hours after insemination 88% of control oocytes and 85% of vitrified oocytes had developed to the 2-cell stage. Only 1.2% of the slow-frozen oocytes had developed. Rates of development to the morula, expanded blastocyst and

Table I. Development of Slow-Frozen and Vitrified MII Mouse Oocytes Following Thawing and In Vitro Fertilization

Groups	No. of oocytes/embryos (%)							
	Cryopreserved	Survival	Inseminated	Fertilized	2 cell	M	Exp. B	H.B
Non-frozen control	—	—	260	241 (92.6)	228 (88)	127 (49)	86 (33)	69 (26.5)
Slow-frozen	294	250 (85)	250	228 (91.2)	3 (1.2) ^{a,b}	0 ^{a,b}	0 ^{a,b}	0 ^{a,b}
Vitrified	203	162 (80)	162	148 (91.4)	138 (85)	47 (46)	52 (32)	44 (27)

Note. M, morula; Exp. B, expanded blastocyst; HB, hatched blastocyst.

^a Indicates significance for slow frozen vs. control ($P < 0.001$).

^b Indicates significance for slow frozen vs. vitrified ($P < 0.001$).

Fig. 1. Ultrastructure of non-frozen control mouse oocyte a-d; at MII and e-f at pronuclear stages. Arrow: microvilli; arrowhead: cortical granules; M: mitochondria; SER: smooth endoplasmic reticulum; L: lipid droplets; Mb: multivesicular bodies; If: intermediate filaments; Lb: laminar body. Xa: 9600; Xb: 34000; Xc: 18000; Xd: 7400; Xe: 24000, Xf: 85000.

hatched blastocyst stages were 49, 33 and 26.5% for the control oocytes and 46, 32 and 27% for the vitrified oocytes. None of the slow-frozen oocytes developed beyond the 2-cell stage. There were no significant differences in rates of development between the vitrified and control oocytes.

Ultrastructure of the Non-Frozen Control Mouse Oocyte

In the non-frozen control MII oocytes, the zona pellucida consisted of closely packed electron dense

fibrillar material. The subzonal space was uniform. The oolemma was covered with long, thin normal microvilli (Fig. 1a,b), except in the zone of polar body extrusion where microvilli were absent. The cortical granules had an electron dense appearance and some were located on the periphery of the ooplasm (Fig. 1a,b), while others were deeper between the other organelles. The cell organelles were distributed throughout the cytoplasm. The mitochondria were usually spherical or oval shaped and their cristae were irregularly placed on the periphery and parallel to the outer mitochondrial membrane. A

large clear vesicle occupied a part of the matrix (Fig. 1c). Mitochondria were more frequent in the cell cortex, particularly adjacent to the lipid droplets, than in the center of the cell. The lipid droplets with no defined membrane aggregated in clusters scattered throughout the cytoplasm (Fig. 1d). The smooth and rough endoplasmic reticulum were also distributed among the other cell organelles. Multivesicular bodies in large numbers and different sizes were seen close to the plasma membrane. Their vesicles appeared light and some had a granular or dense appearance (Fig. 1e). Longitudinal and cross sections of the intermediate filaments of cytokeratin were observed throughout the cytoplasm (Fig. 1a,b,c). These intermediate filaments had a striated appearance. Several crystals in the shape of lamellar bodies, with a configuration of concentric circles and appearance of dark and light lines were occasionally observed throughout the cytoplasm (Fig. 1f). Ten hours post-insemination, oocytes at the pronuclear stage showed similar ultrastructure to that described for the MII stage. However, in comparison with the MII stage, the subzona space was expanded and the cortical granules were less numerous in fertilized non-frozen control oocytes.

Ultrastructure of the Slow-Frozen Thawed Mouse Oocyte

The ultrastructural characteristics of slow-frozen thawed MII oocyte had some similarity to that of the control group, but there were some differences in the morphology of the zona pellucida, intermediate filaments, oolemma and mitochondria. In the zona pellucida; irregularities, fractures, and changes in electron density were occasionally observed (Fig. 2a).

The oolemma was smooth and microvilli were absent (Fig. 2a), however some bleb like projections were occasionally seen (Fig. 2b).

The number of cortical granules in the ooplasm was reduced (Fig. 2b), and the subzonal space was expanded. The intermediate filaments were much less evident throughout the ooplasm. The mitochondria were observed frequently in the central region rather than the periphery of the ooplasm (Fig. 2c). The multivesicular bodies had a different density and some of them had a granular appearance. The lamellar bodies were also seen close to the multivesicular bodies.

Ten hours post-insemination, slow-frozen oocytes at the pronuclear stage had numerous vacuoles throughout the ooplasm (Fig. 2d,e). The mitochondria were much fewer, swollen and had

lost their matrix density (Fig. 2f). The destruction of intermediate filaments was prominent. The subzonal space was not uniform and in some places was dilated. A few cortical granules were still observed throughout the ooplasm. The oolemma, in some regions was still smooth, whereas in the other parts it had some cytoplasmic blebs and a few long loop-shaped microvilli (Fig. 2d).

Ultrastructure of the Vitrified-Thawed Mouse Oocyte

The ultrastructural characteristics of the vitrified-thawed MII oocytes had much similarity to that of the control oocytes. However, the most notable feature of this group after thawing was destruction of the intermediate filaments throughout the ooplasm. The cytokeratin elements were disorganized, while the microvilli which were stabilized by actin filaments had normal morphology similar to that of the control group (Fig. 3a,b). The peripheral cortical granules were prematurely released into the subzonal space, and reduced in number (Fig. 3b). Some of the organelles such as mitochondria and cisternae of smooth endoplasmic reticulum were close together as organelle clusters (Fig. 3c,d). This phenomenon may be because of the disorganization of intermediate filaments. Multivesicular bodies and lipid droplets were also observed throughout the ooplasm (Fig. 3a,c). Ten hours post-insemination, vitrified oocytes at the pronuclear stage showed similar ultrastructure as described for the control group. The cytokeratine intermediate filaments were reorganized (Fig. 3e). The mitochondria were similar to those of the control oocytes (Fig. 3e), and occasionally were observed close to the multivesicular bodies (Fig. 3f).

DISCUSSION

In the present study, the survival and fertilization of the slowly frozen thawed oocytes were similar to that of the vitrified and control oocytes but their developmental capacity to hatching blastocyst was significantly lower. After slow-freezing and vitrification of MII mouse oocytes 1.3 and 93.2%, respectively of one cell embryos had developed to the two cell stage. None of the slow-frozen oocytes developed to the blastocyst stage while 35.1 and 35.7%, respectively of the vitrified and the control oocytes had developed to this stage.

Fig. 2. Ultrastructure of slow-frozen thawed mouse oocyte. a–c; at MII stage, d–f; at pronuclear stage. Arrow: microvillus; arrow head: cortical granules; SO: smooth oolemma; FZ: fractured zona; M: mitochondria; L: lipid droplets; If: intermediate filaments; V: vacuoles. Xa,b: 34000, Xc: 20400, Xd:18000, Xe: 12000, Xf: 60000.

The present study demonstrated that in spite of high survival and fertilization rates slow-frozen oocytes had no developmental potential. Similar findings have been reported by others. Gook *et al.* (2) showed that after slow freezing, using PROH as cryoprotectant, mouse oocytes had a morphological survival rate of 4% and fertilization rate of 0% while human oocytes had a higher survival rate of 64–65%. Al-hasani *et al.* (27) recorded that 75% of human oocytes slowly frozen with PROH as cryoprotectant could be fertilized and in these oocytes 40%

polyploidy was observed. However, there have been reports of full term fetuses resulting from human oocytes frozen by this technique (27,30). These reports suggest that mouse oocytes are more sensitive than human oocytes to slow freezing using PROH as cryoprotectant.

Recently several studies have proven the efficiency of ethylene glycol as cryoprotectant in the vitrification of embryos and oocytes (12,29,31). EG was used first as an embryo cryoprotectant by Miyamoto and Ishibashi (32). Hotamisligil *et al.* (13) reported

Fig. 3. Ultrastructure of vitrified-thawed mouse oocyte. a–d; at MII stage, e–f; at pronuclear stage. Arrow: microvillus; arrow head: cortical granules; SER: smooth endoplasmic reticulum; M: mitochondria; L: lipid droplets; If: intermediate filaments; Mb: mutivesicular body. Xa: 11900, Xb: 85000, Xc: 7400, Xd: 34000, Xe,f: 32500.

that EG was an effective cryopreservation for mouse oocytes. The results of the present study confirm the efficacy of EG as a cryoprotectant for vitrification of mouse oocytes.

Vincent *et al.* (20) reported that exposure of mouse oocytes to cryoprotectants causes release of cortical granules and the zona reaction. Ultrastructural evidence of premature cortical granule release from human oocytes exposed to either PROH or DMSO at room temperature was reported by Schalkoff *et al.*

(33). Subsequent alteration in the zona pellucida may be associated with the low fertilization rate of frozen oocytes. However, the results of this study showed that the fertilization rates of both groups of cryopreserved oocytes were the same as the control group. This result suggests that release of a small portion of the cortical granules may not be harmful to the chances of fertilization.

The ultrastructural investigations in the present study can be divided into two categories: the

structural changes after thawing and at the early fertilization stages. In the slow-frozen oocytes microvilli were absent, the cell membrane was smooth and some mitochondria were swollen whereas the other structures were similar to those in the control oocytes. Most ultrastructural features of the vitrified oocytes were similar to the control oocytes but intermediate filaments were not visible and lipid inclusions were dispersed in the ooplasm. After fertilization, the morphology of the vitrified fertilized oocytes was similar to that of control oocytes; the intermediate filaments had reorganized and had the same morphology as that of the control oocytes. The fertilized slow-frozen oocytes showed severe vesiculation in their cytosol, which may correspond to mitochondria or SER. These oocytes showed other signs of degeneration. The cell membranes of slow-frozen oocyte were smooth and after fertilization some loop shaped microvilli were observed. This may be because microfilaments, which are the cytoskeletal structure of the microvilli (34), are more sensitive to slow freezing than to vitrification. It can be suggested that the MII mouse oocytes were more sensitive to slow freezing than to vitrification. However, our ultrastructural observation in the vitrified oocytes were in contrast with the other investigators who mentioned that the mitochondria were affected by vitrification (25,26). Hochi *et al.* (25) reported that vitrification induced some ultrastructural changes, such as the swelling of mitochondria together with reduced matrix density. The results of the present study showed that the integrity of mitochondria was better preserved in the vitrified than in the slow-frozen oocytes.

In conclusion, the survival and fertilization rates of slow-frozen and vitrified oocytes were the same, but the developmental capacity of the slow-frozen oocytes was low. The ultrastructure of MII mouse oocyte were better preserved by vitrification using EG than by slow freezing using PROH. The cytoskeletal changes of vitrified oocytes is reversible after 10h incubation and no harmful effect can be seen on the developmental capacity of vitrified oocytes.

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