Changes to the meiotic spindle and zona pellucida of mature mouse oocytes following different cryopreservation methods

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Abstract

This study is to investigate the change of morphology of the meiotic spindle and the extent of zona hardening relating to the morphological survival and developmental competence of thawed oocytes. Four- to 8-week-old female mice (C57BL/6) primed with an intraperitoneal injection of pregnant mare's serum gonadotropin and human chorionic gonadotropin. Cryopreserved oocytes using two protocols: vitrificaton using ethylene glycol (EG) and slow freezing using propanediol (PROH). The freezing oocytes were thawed and were fertilized and subsequently cultured in vitro. Spindle/chromosome imagery, dissolution of zona pellucida, and post-thawing survival and development were comparable between two groups. The vitrification cryopreservation method proved to be better than the slow-freezing protocol when comparing the frequency of normal-shaped spindle development post-thawing. The difference in the time required for the dissolution of the zona pellucida under treatment of pronase that was determined to exist between the two cryopreservation methods was statistically significant ($P < 0.005$). The survival rate of post-thawed mature oocytes was significantly greater for the vitrification group than it was for the slow-freezing cryopreservation group ($P = 0.005$). The vitrification cryopreservation of mature murine oocytes would appear to be more satisfactory than the slow controlled-rate freezing method as

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regards the post-thawing oocyte survival and also the incidence of the normal spindle apparatus in the ooplasm.

1. Introduction

Opposing the relatively good results of embryo cryopreservation investigations recently, oocyte cryopreservation techniques, however, especially for human beings, appear to have been frustrated presenting often inconsistent and unstable success rates although good oocyte survival rates, fertilization rates and cleavage rates as well as a few successful pregnancies have been reported in the past (Chen, 1988; Porcu et al., 1997; Yang et al., 1998; Quintans et al., 2002). Till now, it is estimated that approximately 150–200 babies have been born from frozen human oocytes (Tucker et al., 2004).

The process of cryopreservation can contribute to the death of oocytes, parthenogenetic activation (Gook et al., 1995), chromosomal anomaly (Shaw et al., 1991; Park et al., 1997) and perturb the normal activity of the cells' microtubules (Gook et al., 1993; Baka et al., 1995) as a direct consequence of intracellular ice-crystal formation, rapid or massive osmotic and temperature change and the relative toxicity of cryoprotectant under certain conditions (Damien et al., 1990; Hunter et al., 1991, 1995). A too-long exposure time for cells to cryoprotectant will more substantially alter intracellular pH as well as the subsequent post-thawing developmental potential of the cryopreserved cell than would be the case for a shorter exposure time, whereas a too-short exposure time of oocytes to cryoprotectant may induce incomplete dehydration (Damien et al., 1990). Further, the fertilization process and subsequent cleavage can also be impaired by cryopreservation (Hunter et al., 1991, 1995). It has been previously reported that, on occasion, the chromosomes of mature oocytes tended to clump together or were dislocated in the cortical ooplasm following the depolymerization of the spindle microtubules when such mature oocytes were cryopreserved (Sathananthan et al., 1988). In 1990, Pickering et al. (1990) reported that irreversible disorganization of spindle microtubules and chromosomal dispersal in unfertilized oocytes cooled from 37 °C to only room temperature over a period of only 10 min, could occur under certain conditions. It has also been reported, however, that the depolymerized spindle was able to recover subsequent to recuperation of thawed mammals' oocytes at 37 °C for a certain period of time (Eroglu et al., 1998; Chen et al., 2001; Aigner et al., 1992; Aman and Parks, 1994).

It has been proposed that freezing procedures may induce premature cortical granules release from ooplasm, resulting in zona hardening which thus may impair the subsequent fertilization process (Gulyas and Yuan, 1985; Vincent et al., 1990). Although, such a mechanism is challenged in an alternative study, which showed that the inhibition of fertilization that resulted from the freezing process occurred, undoubtedly, primarily at the level of the zona pellucida (Wood et al., 1992). Significant hardening of the zona of mature mouse oocytes was also observed following a "slow-freezing–rapid-thawing" cryopreservation procedure as compared to freshly ovulated oocytes (Matson et al., 1997).

Owing to inherent species differences regarding the cytoskeleton of oocytes, the extent of damage to spindle microtubules and attached chromosomes that may occur during cryopreservation, especially by application of a slow-freezing process, would appear to be quite different. The very poor post-thawing survival and subsequent fertilization rate, 4% and 0%, respectively,

as a consequence of oocyte exposure to a slow cryopreservation method has been previously reported (Gook et al., 1993). However, a recent study has reported an impressive result as regards the successful cryopreservation of human oocytes by the use of a slow-freezing–rapid-thawing protocol (Fabbri et al., 2001). For that study, the oocytes were cryopreserved in a cryopreservation solution consisting of 1,2-propanediol (PROH) accompanied by a greater-than-normal concentration of extracellular cryoprotectant. Post-thawing, the oocytes were inseminated by intracytoplasmic sperm injection (ICSI), and the subsequent outcomes of insemination, embryo development and pregnancy rates were quite inspiring (Fabbri et al., 2001). By comparison to the method of slow, controlled-rate freezing, however, vitrification cryopreservation can simplify the freezing procedure, avoid the need for the acquisition of expensive freezing equipment and reduce the overall need for, and the cost of, an attendant embryologist's time. Besides, there are a few babies already born from vitrified human oocytes (Kuleshova et al., 1999; Kuleshova and Lopata, 2002; Liebermann and Tucker, 2002; Liebermann et al., 2003; Yoon et al., 2003). Establishing simple, efficient freezing and thawing procedures to achieve a better and more consistent result for oocyte cryopreservation is thus clearly warranted.

The aims of the present study are therefore to (i) disclose the influence of a slow controlled-rate oocyte freezing protocol and a vitrification procedure on the chromosomal integrity and the morphology of the oocytes' meiotic spindle, (ii) evaluate the extent of the hardening of the zona pellucida of post-thawed oocytes by using a technique involving the enzyme-digestion of the zona pellucida, and (iii) analyze the morphological survival and developmental competence of cryopreserved and thawed oocytes when the cryopreservation was conducted according to two different cryopreservation methods.

2. Materials and methods

2.1. Mature metaphase II oocyte recovery

Ovulated mature oocytes were collected from 4- to 8-week-old female mice (C57BL/6; Animal Resource Center, National Science Council, Taiwan, ROC) primed with an intraperitoneal injection of 7.5 IU of pregnant mare's serum gonadotropin (PMSG, Sigma, G-4877; The Sigma Chemical Co., St. Louis, MO, US), and followed by the administration of 7.5 IU of human chorionic gonadotropin (hCG 2000 IU, Serono, Singapore) 42–48 h later intraperitoneally. Sacrifice of the female mice 10–13 h post-hCG injection. The oviducts were excised, and the cumulus–oocyte-complexes were collected in human tubal fluid (HTF) medium. Denuding of the cumulus was performed with 80 IU/ml hyaluronidase (Sigma, H-3757, US, 307 IU/mg, 100 mg). The harvested oocytes were cultured in HTF medium (Irvine, 90125, US) containing 10% fetal cord serum at 37 °C in an incubator featuring an atmosphere of 5% CO_2 in air.

2.2. Cryopreservation of oocytes

2.2.1. PROH slow-freezing/rapid-thawing method
Oocytes from the slow-frozen group were frozen using the slow-freezing method reported elsewhere (Fabbri et al., 2001). Briefly, all the harvested oocytes were transferred to petri dishes containing Dulbeco's phosphate buffered solution (PBS) (Irvine, 9236, US) supplemented with 10% fetal cord serum (FCS) and maintained at room temperature following incubation for a period of 2–3 h at 37 °C, 5% CO_2. The fetal cord serum was prepared from the heat-inactivated serum deriving from human umbilical cord blood. Oocytes were exposed to 1.5 M PROH in phosphate

buffered saline supplemented with 30% FCS for 10 min, then transferred to 1.5 M PROH and 0.3 M sucrose in phosphate buffered saline supplemented with 30% FCS for 10 min. The oocytes were then loaded into 9-cm plastic straws (Paillette Cristal, CBS 1623, France) and transferred into an automated Kryo 10 series III freezer. The temperature was gradually decreased to $-7\,^\circ\text{C}$ ($2\,^\circ\text{C/min}$) at which point seeding of the straws was induced. After 5 min, the temperature was lowered to $-30\,^\circ\text{C}$ at the rate of $0.3\,^\circ\text{C/min}$. The straws were held at this temperature for 30 min. The oocytes were then rapidly cooled to $-196\,^\circ\text{C}$ and stored in liquid nitrogen. After 2 weeks straws were thawed by transfer from liquid nitrogen to room temperature for 30 s, followed by shaking in a $30\,^\circ\text{C}$ water bath for 30 s. Cryoprotectant was removed by passing the oocytes through decreasing of PROH (1 M, 0.5 M, 0.25 M, and 0 M) in 0.3 M sucrose in phosphate buffered saline supplemented with 30% FCS (5 min for each step). Finally, the oocytes were washed twice in phosphate buffered saline, scored for survival and transferred to a drop of HTF medium containing 10% FCS.

2.2.2. Vitrification and thawing procedure

Oocytes of the vitrified group were frozen using the vitrification method reported elsewhere (Chen et al., 2000). Briefly, vitrification solution was prepared using 1.5 mol/l ethylene glycol (EG; Sigma, E-9129, US) in PBS with 20% FCS (EG 1.5) and EG 5.5 mol/l in PBS with 20% FCS and 1.0 mol/l sucrose (EG 5.5).

The oocytes were transferred to EG 1.5 at room temperature and held for 10 min. Then transfer to EG 5.5 for about 1 min. Groups of 15–20 oocytes were loaded into 0.5 ml straws (Paillette Cristal, CBS 1623, France). 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 $25\,^\circ\text{C}$ water bath for 25 s. The contents of each straw were expelled into 0.8 ml of 0.5 M, 0.25 M, and 0.125 M sucrose in phosphate buffered each for 2.5 min. The oocytes were washed twice in phosphate buffered saline, scored for survival and transferred to a drop of HTF medium containing 10% FCS.

2.3. Dissolution of zona pellucida

The zona was removed using a 20 IU/ml solution of pronase (5.6 IU/mg; 100 mg; Sigma, P-8811, US) dissolved in PBS. The procedure was performed on an inverted microscope (Olympus, IMT-2, Japan) fitted with a stage warmer set at $37\,^\circ\text{C}$. The dissolution time for the zona pellucida was recorded as having been completed when its border was no longer clearly defined under $200\times$ magnification subsequent to treatment with pronase.

2.4. Spindle and chromosome imagery

After 60-min of in vitro culture, the post-thaw oocytes were sent to spindle and chromosome studies. After treatment with pronase at $37\,^\circ\text{C}$, zona-free oocytes were attached to poly-L-lysine-coated (1–2 mg/ml; Sigma, P-1524, US) coverslips and fixed in 2% formaldehyde (Sigma, US) with 0.02% Triton X-100 (Sigma, US) in PBS (pH 7.3) for a period of 30 min. Fixed oocytes were then permeated for 40 min with PBS containing 0.1% Triton X-100. In order to attempt to reduce any remaining free aldehydes which may have been present and which might bind primary and/or secondary antibodies, thus increasing non-specific background fluorescence, oocytes were rinsed for 30 min in PBS containing 50 mM glycine (Sigma, G-6761, US) and 3 mg/ml bovine serum albumin (BSA; Sigma, A-4503, US). Spindle microtubule localization was performed by

adding a mouse monoclonal antibody to β-tubulin (Sigma, F-2043, US) diluted 1:10 in 0.5% BSA in a PBS solution on a glass slide for a period of 45 min at 37 °C. Subsequent to washing in a solution of PBS–0.01% Triton X-100 containing 0.5% BSA, the primary antibody was detected using a fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse IgG secondary antibody (Sigma, F-5387, US) diluted 1:32, and which was incubated with the oocytes for a further 45 min at 37 °C. Chromosome detection was achieved by incubating fixed oocytes in DAPI (5 μg/ml in PBS, Sigma, D-9542, US) at room temperature for a period of 10 min.

Coverslips were wet mounted using an antifade medium (SlowFade; Molecular Probes; Sigma, US) in order to retard photobleaching. Spindle morphology and DAPI-staining chromosomes were observed by fluorescent microscopy (LEICA, type 020-519-511, DMLB 100T) with a variety of different filter settings being adapted. Photographs were taken using appropriate software of Spot Basic. Adobe Photoshop software (Version 6.0) was used for the subsequent processing of pictures.

2.5. Collection of sperm and subsequent insemination procedure

Spermatozoa were extracted from the cauda epididymis of CBA mice and capacitated for 1.5 h in HTF medium containing 10% FCS. The surviving MII oocytes from both experimental groups and the control non-frozen oocytes were transferred to HTF medium containing 10% FCS, inseminated with 100,000 capacitated sperm per oocyte and incubated in a humidified 37 °C incubator in an atmosphere of 5% CO_2 in air. The culture medium was renewed with KSOM-aa medium after 4–6 h. Two-cell embryos were observed at the second day.

2.6. Statistics

The statistical package for social sciences (SPSS, Version 10.0; SPSS Inc., Chicago, IL, US) software was used in order to examine the experimental results. The data pertaining to oocyte survival, normal embryo development and normal spindle incidence were analyzed using a Pearson chi-square test or Fisher's exact test in order to determine whether significant difference arose between control and experimental groups. As regards the comparison of the time required for zonal digestion between control and the two experimental groups, a Kruskal–Wallis non-parametric test method was adopted in order to determine whether difference between these test groups existed at a statistically significant level. Difference was considered to constitute significance if $P < 0.05$.

3. Results

3.1. Spindle morphology analysis

The results of spindle analysis were summarized in Table 1. We classified the spindle morphology (Fig. 1) as being characteristic of membership of one of the four different groups: (i) classic barrel-shaped spindle appearing with fine microtubules traversing the metaphase plate, normally situated tangentially to the oolemma and with the chromosomes being located at the equatorial region (A–C), (ii) activated telophasic spindle of a barrel or a tapered shape with rotation, situating radially to the oolemma and featuring chromosomal condensation and their segregation into the bipolar regions (D–H), (iii) teardrop-shaped or tapered spindle featuring a reduction in spindle length with clumped or dispersed and disorganized chromosomes (I–N), (iv) the presence of a

Table 1
Pattern of spindle appearance amongst cryopreserved mature murine oocytes subsequent to a 1-h post-thaw incubation period

Outcome	Group		
	Control 30 ($n = 3$)[a]	PROH slow protocol 39 ($n = 5$)[a]	Vitrification protocol 45 ($n = 4$)[a]
Normal barrel-shape	27 (90%)[b,d]	16 (41%)[b,c]	28 (62.2%)[c,d]
Activated shape	3 (9.9%)	15 (38.5%)	10 (22.2%)
Teardrop-shape	0	2 (5.1%)	7 (15.6%)
Disappearing	0	6 (15.4%)	0

PROH = 1,2-propanediol protocol.
[a] n = number of experiments.
[b] $P < 0.001$.
[c] $P = 0.05$.
[d] $P = 0.008$ (all by Pearson chi-square test).

disappearing spindle but featuring separate fragmentary condensed or ring-shaped chromosomes as revealed by staining (O–R).

For the control group, around 90% of the unfertilized mature oocytes presented as a normal barrel-shaped spindle, with chromosomes being condensed and located at the metaphase equatorial plate. These results disclose that the rate of appearance of the normally shaped spindle following
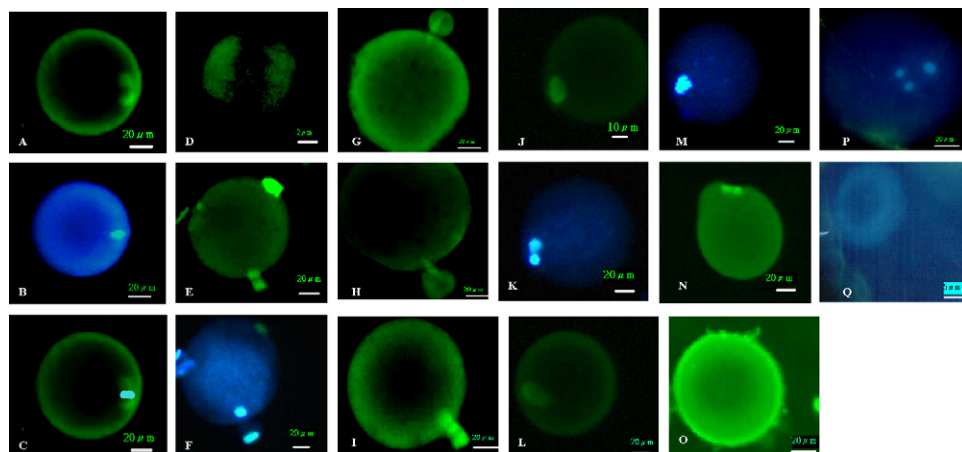

Fig. 1. Fluorescent micrographs of the meiotic spindle and the chromatin deriving from unfertilized mature mouse metaphase II oocytes and as indicated by immunofluorescent staining techniques—(A and B) control unfrozen mature metaphase II oocytes: a normal barrel-shaped spindle appears to be located tangentially to the oolema surface and clustered metaphase II chromosomes are located at the equatorial plate (200×); (C) enlarged view of a normal barrel-shaped spindle (400×); (D–H) cryopreserved oocyte revealing a polar-body extrusion (not visible subsequent to the zona digestion and staining procedures except (F) and (G) with an activated telophasic barrel- or tapered shape spindle, situating radially to the oolema and featuring chromosomal condensation and segregation into the bipolar region (D and E: 200×; F–H: 400×); (I–N) cryopreserved oocytes revealing a teardrop-shaped or a tapered spindle within the ooplasm with a reduced spindle length and featuring dispersed and disorganized chromosomes (200×); (O–Q) cryopreserved oocytes revealing a disappearing spindle featuring separating yet condensed or abnormal ring-shaped chromosomes (400×); (R) enlarged view of (Q). Note the whitish bar: 20 mm in length under 200× magnification; the yellowish bar: 15 mm in length under 400× magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2

The time required for the dissolution of the zona pellucida of mature murine oocytes (P value < 0.001)[a]

	Dissolution time (s)
Group	
Control	123 ± 21 ($n = 10$)*
PROH slow protocol	1276 ± 292 ($n = 10$)*
Vitrification protocol	590 ± 176 ($n = 10$)*

Note: Values are mean $\pm$ S.D.; n: number of oocytes. PROH = 1,2-propanediol protocol.

[a] Non-parametric test by Kruskal–Wallis method.

both the slow-freezing PROH (41%) and the vitrification (62.2%) protocol were statistically significantly lower than was the case for the control group. The vitrification method appeared to be more satisfactory than the slow PROH protocol when comparing the rate of presentation of a normal-shaped spindle ($P = 0.05$). By determining and analyzing the level of abnormality of spindle shape for the mature oocytes subsequent to the freezing–thawing procedure, we were able to determine that there appeared to be a greater incidence of the appearance of an activated spindle and a disappearing spindle when using the slow PROH protocol as compared to vitrification protocol.

3.2. "Zona hardening" analysis

We observed that it typically required exposure of the unfertilized mature oocytes to pronase (20 IU/ml) for a period of about 2 min in order to achieve complete dissolution of the zona pellucida. The results presented in Table 2 revealed the influence of previous cryopreservation upon the dissolution time of the zona pellucida. It would appear that the freezing–thawing process exerts a substantial influence upon zona hardening. These results indicated that a statistically significantly greater period of exposure of oocytes to pronase was required in order to digest the zona pellucida of post-cryopreserved and thawed murine oocytes by either of the two different cryopreservation methods, than was the case for control oocytes (Table 3). The mean time required for the digestion of the zona pellucida was around 20 min for oocytes cryopreserved by the PROH slow-freezing cryopreservation technique and thawing procedure whilst the corresponding time was only around 10 min for the vitrification method. The corresponding difference in dissolution time, using pronase (20 IU/ml), for the zona pellucida for oocytes previously cryopreserved by the two different cryopreservation techniques was also noted to be statistically significant ($P < 0.005$).

Table 3

The morphological survival of mature metaphase II oocytes subsequent to the cryopreservation/thawing process and subsequent normal two-cell stage embryo formation by in vitro insemination

Outcome	Group		
	Control 30 ($n = 2$)	PROH slow protocol 30 ($n = 3$)	Vitrification protocol 27 ($n = 3$)
Survival following cryopreservation		10 (33%)[a]	19 (70%)[a]
Normal two-cell stage embryos resulting	21 (70%)	2 (20%)[b]	5 (26.3%)[b]

PROH = 1,2-propanediol protocol.

[a] $P = 0.005$ (by Pearson chi-square test).

[b] $P = 1$ (by Fisher's exact test).

3.3. Survival and developmental competence of oocytes (Table 3)

The survival of oocytes was determined by observing the oocyte morphology as regards the presentation of regular oocyte shape and diameter, the presence of an intact zona pellucida and oolemma, a clear perivitelline space, normal oocyte size and no evidence of ooplasmic degeneration. The morphological survival rate of mature oocytes was 33% for the PROH slow-freezing cryopreservation technique and 70% for the vitrification cryopreservation protocol. The survival rate of post-thawed mature oocytes was significantly higher for the vitrification group than it was for the PROH slow-freezing cryopreservation group ($P = 0.005$). We also investigated the developmental competence of surviving oocytes following the applied cryopreservation procedures by means of the determination of the oocytes' potential for in vitro fertilization. On the day following in vitro fertilization, about 18–20 h after insemination, the number of normal two-cell stage embryos were enumerated. Unlike the oocyte survival rate, the normal two-cell stage embryo formation rate did not differ statistically significantly between the two different cryopreservation methods.

4. Discussion

This study presented a comparison of the morphological survival and developmental competence of murine mature metaphase II oocytes following their passage through two different cryopreservation procedures. The spindle and chromosome morphology were analyzed by using immunofluorescent techniques and the dissolution of the zona pellucida was determined by use of an enzyme-digestion method.

For the purposes of achieving good fertilization and further cleavage ability, mature oocytes must reflect normal cytoskeletal organization, for example, the normal spindle structure anchoring with ordinarily aligned chromosomes. This is especially the case for oocytes subsequent to a cryopreservation process, such treatment is frequently associated with the disassembly of cell microtubules resulting in the abnormal shape or the destruction of the spindle apparatus (Trounson, 1990; Aigner et al., 1992; Joly et al., 1992; Park et al., 1997). Disorganization or disappearance of the meiotic spindles of cryopreserved oocytes would bring about aneuploidy or polyploidy after fertilization (Sathananthan et al., 1987; Eroglu et al., 1998). If the spindles were completely disintegrated, without extrusion of the second polar body, polyploidy with one female pronucleus and one male pronucleus may occur. Digyny with two female pronuclei and one male pronucleus indicated segregation of the maternal chromosomes to a certain extent by the compromised meiotic spindle. The injured microtubules to a lesser degree may cause formation of micronuclei from dispersed chromosomes or aneuploidy (Sathananthan et al., 1987). However, some abnormal spindles in the post-thawing oocytes could recover to normality following a certain period of recuperation under suitable culture conditions (Chen et al., 2003). Incubation for 1–3 h at 37 °C resulted in recovery of spindles in diverse degrees that may be dependent on time intervals after thawing, methods of freezing, and species (Aigner et al., 1992; Gook et al., 1993; Chen et al., 2001). Eroglu et al. (1998) noticed complete recovery of spindles for slow freezing mouse oocytes after incubation for 1 h post-thawing. Using ultra-rapid or vitrification methods in mouse oocytes, incubation for 2 h or 3 h resulted in higher incidences of normal spindles than incubation for 1 h (Aigner et al., 1992; Chen et al., 2001). Therefore, reducing the injury and permitting recovery is critical for cryopreservation of oocytes. Our experimental results revealed the incidence of an abnormally shaped spindle in post-thawing oocytes was significantly higher in both slow controlled-rate freezing and vitrification cryopreservation groups than unfrozen control group ($P < 0.001$ and $P = 0.008$, respectively).

The time required for the effective dissolution of the zona pellucida by the use of the enzyme, α-chymotrpsin was determined herein, in order to evaluate the extent of zona "hardening" arising as a consequence of the mature oocyte having undergone a freezing–thawing procedure (Matson et al., 1997). Our study demonstrated that, for the zona pellucida of unfertilized mature oocytes, the time required for its complete dissolution was about 5 min (data not shown) in pronase at a concentration of 10 IU/ml and about 2 min at a pronase concentration of 20 IU/ml. The effect of zona hardening post-cryopreservation and thawing appeared to be very pronounced for the mature oocytes studied herein. Such a characteristic change was considered to introduce a significant handicap to the fertilization process of previously cryopreserved and thawed oocytes especially when conducted via process of in-vitro insemination with sperm. The possible mechanism underpinning failed fertilization under such circumstances is that the penetration of the hardened zona typically depletes the energy reserves of the sperm and modifies the binding sites on the sperm head (Wood et al., 1992).

The morphological survival of embryos or oocytes subsequent to a freezing–thawing procedure depended upon several factors including change of osmotic pressure, intracellular and extracellular ice formation (Ashwood-Smith et al., 1988). Cell damage that is due to intracellular ice formation cannot be excluded from either the slow controlled-rate freezing or rapid-rate freezing procedures (Van den Abbeel et al., 1997). The rapid-thawing process adopted for both cryopreservation methods attempted herein was selected in order to reduce the possible reformation of tiny intracellular ice-crystals that might have been formed during the freezing process as a consequence of the incomplete dehydration of the oocytes (Trounson, 1986). We suggest that the differences in morphological survival of cryopreserved and thawed oocytes for the slow and the vitrification cryopreservation methods as reported herein probably resulted as a consequence of whether extracellular ice-crystals arose or not. However, it has been demonstrated herein that morphological survival was not necessarily a very good indicator as regards determining the efficiency of a cryopreservation procedure (Van den Abbeel et al., 1997). The authors reported that the cleavage rate of morphologically intact post-cryopreservation and thawing embryos was higher following a slow-freezing process than it appeared to be subsequent to a rapid-freezing process (Van den Abbeel et al., 1997).

In the present study, the morphological survival rate of thawed oocytes was significantly higher for the vitrification group than it was for the slow cryopreservation group. On the other hand, the developmental competence of the fertilized oocytes following cryopreservation did not appear to differ significantly for oocytes that had been cryopreserved by either of the cryopreservation methods, although the incidence of a normal spindle was significantly lower for the slow-cryopreservation group. The mean time required for zona dissolution for oocytes having passage either of the two freezing–thawing protocols was statistically significantly greater than unfrozen mature oocytes. It appears likely that fertilization via the sperm–zona interaction should be considerably impaired under such an impact. In fact, we suggest that the influence of cryopreservation upon fertilization should be exerted much more at the zona level than at the cytoskeleton level, it has been substantiated that the disorganized microtubules of cryopreserved and thawed cells were able to recover to some extent subsequent to suitable environment provided for recuperation of post-thawed oocytes.

5. Conclusion

In conclusion, the vitrification cryopreservation of mature murine oocytes was more satisfactory than was the slow controlled-rate freezing method when considering the post-thawed

oocyte survival rate and the normal spindle incidence within the oocyte ooplasm. The subsequent developmental competence of in vitro fertilized oocytes should be able to be improved upon via insemination by intracytoplasmic sperm injection rather than in vitro insemination. As regards the future for oocyte cryopreservation for humans, it still appears that we require a simpler, more reliable, efficacious and steady oocyte-vitrification protocol in order to fulfill this common and increasingly more-important requisite of improving assisted reproductive techniques to help many infertile couples achieve viable pregnancy.

Acknowledgements

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