ANIMAL EXPERIMENTATION

Cytoskeleton and Polyploidy After Maturation and Fertilization of Cryopreserved Germinal Vesicle–Stage Mouse Oocytes

A. EROGLU,^{1,2} M. TONER,² L. LEYKIN,¹ and T. L. TOTH^{1,3}

Submitted: January 2, 1998

Accepted: March 6, 1998

Purpose: Our purpose was to assess the effect of cryopreservation on cytoskeleton of germinal vesicle (GV) mouse oocytes and determine whether irreversible spindle damage and related digyny associated with cryopreservation of metaphase II (MII) oocytes can be avoided.

Methods: The GV oocytes were cryopreserved using a slow-cooling (0.5°C/min) and slow-thawing (8°C/min) protocol in 1.5 M dimethylsulfoxide supplemented with 0.2 M sucrose and analyzed before and during fertilization by multiple-label fluorescence and differential interference contrast microscopy techniques.

Results: When examined after in vitro maturation, the vast majority (>95%) of cryopreserved and control oocytes displayed normal microfilament and microtubule organization. With respect to barrel-shaped spindle and normal chromosome alignment, no significant differences were observed between cryopreservation (78 and 86%, respectively) and control (85 and 95%, respectively) groups. In fertilization experiments, spindle rotation, formation of the second polar body, and pronuclear migration were displayed by similar percentages of cryopreserved (96, 94, and 37%, respectively) and control (98, 97, and 45%, respectively) oocytes, indicating normal functionality of the cytoskeleton during this period. However, pronuclear formation was significantly inhibited by cryopreservation (81%) compared with controls (100%). Regarding digyny and polyspermy, no significant

increase was observed after cryopreservation (3 and 10%, respectively) compared with controls (3 and 6%, respectively).

Conclusions: Cryopreservation of mouse oocytes at the GV stage is particularly advantageous to circumvent the spindle damage and increased digyny noted after cryopreservation of MII oocytes.

KEY WORDS: cryopreservation; cytoskeleton; digyny; fertilization; oocyte; pronuclear formation.

INTRODUCTION

Oocyte cryopreservation may avoid ethical/legal issues associated with human embryo freezing and provide future fertility for women who may lose gonadal function due to extirpative therapy, radiation, or chemotherapy. However, the success rate of human oocyte cryopreservation is still low. Achievement of a clinically feasible technique requires further research to better understand and overcome cryopreservation-associated stresses and problems such as spindle disorganization (1–3), disruption of cytoskeletal elements (3,4), and increased polyploidy (5–7).

Proper organization and function of oocyte cytoskeleton are essential for correct segregation of chromosomes, spindle rotation, polar body (PB) formation, pronuclear formation, and migration (8,9). However, the oocyte cytoskeleton is known to be very sensitive to physiochemical stresses associated with cryopreservation. Studies using metaphase II (MII) human (10,11), mouse (1,12), cow (2), and rabbit (4) oocytes showed that cooling, cryoprotectants, or freezing and thawing cause depolymerization of the MII spindle microtubules with a consequence of chromosomal

¹ Vincent Memorial Obstetrics and Gynecology Service, Massachusetts General Hospital, and Harvard Medical School, Boston, Massachusetts.

² Center for Engineering in Medicine and Surgical Services, Massachusetts General Hospital, and Harvard Medical School, and the Shriners Burns Institute, Boston, Massachusetts.

³ To whom correspondence should be addressed at Massachusetts General Hospital, Vincent Memorial Obstetrics and Gynecology Service, Vincent-2, Boston, Massachusetts 02114.

scattering. In addition, exposure of MII oocytes to cryoprotectants or a freeze-thaw cycle disrupts microfilament network and induces formation of several cytoplasmic microtubular asters or network (3,4). Although these alterations in microfilament and cytoplasmic microtubule organization of MII oocytes can be reversed by a subsequent incubation, changes in the spindle morphology are partially irreversible (3,4,12). The partially irreversible damage seemed to contribute to increased digyny after fertilization (3). Unlike the MII oocytes, germinal vesicle (GV)-stage oocytes do not contain any prominent microtubular structure except a few asters and their chromatin is in a decondensed state within a membrane-bound nucleus (13). Therefore, GV-stage oocytes are theoretically less susceptible to this kind of freezing injury. In fact, this is supported by a recent study showing that human GV-stage oocytes can be matured and fertilized successfully after cryopreservation (14).

The objective of this study was to examine (i) how oocyte cryopreservation at the GV stage affects cytoskeletal organization and function before and during fertilization and (ii) whether irreversible spindle damage and increased digyny observed after cryopreservation of MII oocytes can be avoided by performing cryopreservation at the GV stage. To this end, we have applied the slow-cooling and slow-thawing protocol used in our previous MII study to the GV oocytes and monitored cytoskeletal alterations and polyploidy using triple fluorescence staining and differential interference contrast (DIC) microscopy.

MATERIALS AND METHODS

Oocyte Isolation

Oocytes were obtained from 4- to 6-week-old virgin F₁ hybrid mice (C57BL/6 × DBA/2; Charles River Laboratories, Wilmington, MA). Follicular development was stimulated by intraperitoneal injections of 7.5 IU pregnant mare's serum gonadotropin (PMSG; Sigma, St. Louis, MO). Forty-eight to 50 hr after injection, oocytes were released into Dulbecco's phosphate-buffered saline (PBS; Sigma) containing 4 mg/ml bovine serum albumin (BSA; Fraction V; Sigma) and 0.25 mM dbcAMP (Sigma) by puncturing large antral follicles. Only fully cumulus-enclosed GV oocytes were selected and washed twice before use.

Cryopreservation of GV Oocytes

Cumulus-enclosed GV oocytes were cryopreserved using our slow-cooling/slow-thawing protocol optimized for MII mouse oocytes (3). Briefly, the addition of cryoprotectants was carried out in a cold room (4°C) by keeping oocytes in a freezing medium composed of 1.5 M dimethylsulfoxide (DMSO) (Sigma), 0.2 M sucrose (Sigma), 0.25 mM dbcAMP, and 4 mg/ml BSA in PBS for 30 min. Next, the oocytes were aspirated into plastic straws and then were placed in a programmable freezer (Planer Kryo 10-II; TS Scientific, Perkasie, PA) at 0°C and cooled to -7°C at 2°C/min. After seeding of extracellular ice and holding at -7°C for 15 min, straws were cooled to -80°C at 0.5°C/min and then plunged into liquid nitrogen (LN₂). Oocytes that were stored in LN₂ for 1-7 days were thawed by reintroducing straws into the freezer at -80°C and warming them to 4°C at 8°C/min. Subsequently, the contents of the straws were released into 1 ml PBS containing 0.2 M sucrose, 0.25 mM dbcAMP, and 4 mg/ml BSA at ambient temperature. After 10 min, the oocytes were transferred to 1 ml PBS containing 0.25 mM dbcAMP and 4 mg/ml BSA at ambient temperature for an additional 10 min and then washed in PBS supplemented with 4 mg/ml BSA at 37°C. Evaluation of morphological survival (homogenous cytoplasm, intact plasma membrane and zona pellucida) was carried out after both dilution of cryoprotectants and in vitro maturation.

In Vitro Maturation and Fertilization

All cultures were carried out at 37°C under a humidified atmosphere of 5% CO₂ in air. Before use, large drops (0.5 ml) of culture medium overlaid with silicone oil (Aldrich, Milwaukee, WI) were equilibrated under the same conditions. Waymouth's MB 752/1 medium (Life Technologies, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS; Sigma), 0.33 mM sodium pyruvate (Sigma), 50 mg/ml gentamycin (Sigma), 2.5 IU/ml follicle stimulating hormone (FSH; Sigma), and 1 mg/ml estradiol-17 β served as the in vitro maturation medium. Frozen-thawed and freshly collected GV oocytes were washed through two changes of the in vitro maturation medium and then cultured in the same medium for 17-18 hr. At the end of the culture period, oocytes were denuded by repeated gentle micropipetting in prewarmed (37°C) PBS supplemented with 4 mg/ml BSA. Surviving oocytes were additionally incubated in the in vitro maturation medium at 37°C for 30-40 min before fixation or insemination.

In vitro fertilization (IVF) was carried out in bicarbonate-buffered human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA) supplemented with 5 mg/ml BSA as described in detail previously (3). Briefly, 5 hr after introduction to sperm suspension ($1-2 \times 10^6$ sperm/ml), oocytes were removed from the insemination dishes, washed twice in HTF, and then cultured in the same medium.

Fixation, Fluorescence Labeling, and Microscopy

Fixation and multiple fluorescence labeling were described in detail previously (3). Briefly, oocytes were simultaneously fixed and extracted in a microtubule stabilizing buffer (0.1 M Pipes, pH 6.9, 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.5 mM EGTA, 2.0% formaldehyde, 0.1% Triton X-100, 1 μM Taxol, 0.01% Aprotinin, and 50% deuterium oxide) for 30–40 min at 37°C. Next, oocytes were washed twice in a blocking solution consisting of 0.02% sodium azide, 2% normal goat serum, 2% powdered milk, 2% BSA, 0.1 M glycine, and 0.01% Triton X-100 in PBS and stored in the same blocking solution at 4°C for 1–15 days until labeling. A primary mouse anti- α -tubulin monoclonal antibody (Amersham, Arlington Heights, IL) and a secondary fluorescein-conjugated goat anti-mouse immunoglobulin (Boehringer-Mannheim, Indianapolis, IN) were used to label microtubules. Microfilaments were visualized by rhodamine-phalloidin (Molecular Probes, Eugene, OR), while Hoechst 33342 (Molecular Probes) was used to stain DNA. Labeled oocytes were mounted in a glycerol-based medium containing 1% *n*-propyl gallate as an antifading agent. A Nikon Diaphot 300 microscope (Nikon, Melville, NY) equipped with fluorescein, rhodamine, and Hoechst selective filter sets and a 100-W superhigh-pressure mercury arc lamp was used for DIC and fluorescence microscopy of specimens. The images were acquired using a Hamamatsu SIT camera (C24000) and a Macintosh Quadra 950 with Signal Analytics IPLab Spectrum software. Acquired images were processed using Adobe Photoshop 3.0 and Aldus Persuasion 3.0 software packages and printed on a Tektronix Phaser IISDX printer.

Prefertilization Experiments

Freshly isolated cumulus-enclosed GV-stage oocytes were divided into two groups. One group was first subjected to a freeze-thaw cycle and then cultured for in vitro maturation. The other group served as a control and oocytes were directly cultured without any freezing

manipulation. After culture for in vitro maturation, all oocytes in both groups were fixed to analyze meiotic stage, microfilament and microtubule pattern, spindle shape, and chromosome alignment.

Fertilization Experiments

Frozen-thawed and control GV-stage oocytes which extruded the first polar body (PB1) during culture were selected for insemination. The remaining oocytes, which did not form the PB1, were fixed for analysis without further manipulation. Inseminated oocytes were fixed at 8 hr postinsemination. The introduction of oocytes to sperm suspension was considered as time 0. Eggs were considered fertilized when sperm penetration was clearly determined using DIC and fluorescence microscopy. The spindle was scored as rotated when it was perpendicular to the cell surface. If the border of the second polar body (PB2) was completely distinguishable by f-actin staining, the PB2 was considered formed. The appearance of maternal and paternal chromatins in a round shape was considered indication of pronuclear formation. Pronuclei were considered migrated when male and female pronuclei came together in the egg center. Polyspermy was assessed by the observation of more than one penetrated sperm head or male pronucleus with corresponding sperm tails. Eggs were considered digynic when they displayed two or more pronuclei with one missing sperm tail and either no PB2 or a PB2 exhibiting no DNA staining.

Statistical Analysis

Results were analyzed by Yates-corrected chi-square test. Fisher's exact test was used when one of the expected values in a contingency table was less than five. Differences between groups were considered significant when *P* was less than 0.05. Experiments were repeated at least three times for each condition.

RESULTS

Overall Morphological Survival and In Vitro Maturation

Morphological examination after removal of cumulus cells following in vitro maturation revealed that of the total 277 frozen fully cumulus-enclosed GV oocytes, 200 (72%) survived cryopreservation. The meiotic competence of cryopreserved GV oocytes and

their nonfrozen controls was assessed after a culture period of 17–18 hr. Similar proportions of cryopreserved (99%) and control (100%) oocytes underwent GV breakdown. The percentage of oocytes which progressed to the MII stage was also not statistically different between the cryopreservation (74%) and the control (82%) groups. These results indicate that cryopreservation did not significantly impede the ability of oocytes to mature.

Cytoskeletal Organization During the Prefertilization Period

Figures 1A and B depict typical normal morphologies observed in control and cryopreserved oocytes. Statistical analysis of the data showed that cryopreservation at the GV stage did not cause any significant abnormalities in microfilament and microtubule organization, spindle morphology, and chromosome alignment of oocytes when examined after in vitro maturation (Table I). Only a statistically insignificant portion of oocytes (5–22%) displayed depolymerized spindle, dispersed chromosomes, and no microfilament-rich domain over MII chromosomes (Fig. 1C)

Cytoskeleton-Dependent Events During the Fertilization Period

Both cryopreserved and control oocytes showed a high sperm penetration rate (88 and 89%, respectively),

with no significant difference, indicating that cryopreservation did not negatively affect fertilization (Table II).

Figures 2A and B depict typical normal morphologies observed in control and cryopreserved oocytes, respectively. Statistical analysis of the data revealed that cryopreservation induced no significant failure in spindle rotation, PB2 formation, and pronuclear migration compared to the control group (Table II). However, pronuclear formation was significantly inhibited by cryopreservation (81%) in comparison to the control group (100%), with $P < 10^{-3}$ (Table II). Cryopreserved eggs that failed to form pronuclei exhibited generally two types of aberrant cytoskeletal pattern. The first type of aberrant pattern was displayed by 68% of the eggs that failed to form pronuclei (Fig. 2C). Pronuclear development in these eggs appeared to be arrested at an early stage. The sperm head was in decondensed form and cytoplasmic microtubules appeared as a network. The second type of aberrant pattern was observed in the 32% of the eggs that skipped the pronuclear formation and began to form a spindle around the recondensed sperm nucleus and egg chromosomes (Fig. 2D). The spindle remnant, which is normally still prominent in normal eggs fixed at 8 hr postinsemination, completely disappeared in these eggs. Microtubules appeared as radiating arrays from the recondensed sperm head, egg chromosomes, and chromosomes of PB2, a phase in microtubular organization that normally corresponds to an early stage of spindle formation. Indeed, the spindle forma-

Fig. 1. (*Opposite, top*) Cytoskeletal organization and chromosome alignment of control and cryopreserved mouse oocytes before fertilization. Triple fluorescence staining depicts microfilaments (red), microtubules (green), and DNA (blue). (A) A control oocyte fixed after in vitro maturation. The arrowhead shows the barrel-shaped spindle with chromosomes properly aligned at the equatorial plate. The arrow indicates the outer ring of the microfilament-rich domain. PB1 (arrow-1) is intensely stained for microfilaments and contains a spindle. (B) A cryopreserved oocyte fixed after in vitro maturation. The arrowhead shows a barrel-shaped spindle with chromosomes properly aligned at the equatorial plate. The arrow indicates the outer ring of the microfilament-rich domain. PB1 (arrow-1) is intensely stained for microfilaments and contains a spindle. (C) A cryopreserved oocyte fixed after in vitro maturation displaying a depolymerized spindle and dispersed chromosomes (arrowhead) which are not surrounded by a microfilament-rich domain. The PB1 (arrow-1) is intensely stained for microfilaments and contains a spindle. Bar = 20 μ m.

Fig. 2. (*Opposite, bottom*) Cytoskeletal organization of sperm-penetrated mouse oocytes matured in vitro. Triple fluorescence staining depicts microfilaments (red), microtubules (green), and DNA (blue). (A) A control oocyte fixed at 8 hr postinsemination displaying a male and a female pronucleus migrated to the egg center. Arrow-1 and arrow-2 indicate the PB1 and PB2, respectively. (B) A cryopreserved oocytes fixed at 8 hr postinsemination showing a male and a female pronucleus migrated to the egg center. Arrow-1 and arrow-2 indicate the PB1 and PB2, respectively. (C) A cryopreserved oocyte fixed at 8 h postinsemination showing failure of pronuclear formation. The arrowhead shows a decondensed sperm nucleus. Arrows indicate outer rings of microfilament-rich domains surrounding the decondensed sperm nucleus and maternal chromosomes. (D) A cryopreserved oocyte fixed at 8 hr postinsemination displays the early phase of spindle formation indicating bypass of the pronuclear stage. Arrowheads indicate the almost-recondensed sperm nucleus and maternal chromosomes with radiating microtubular arrays. The PB1 (arrow-1) is dividing and, therefore, stained intensely for microfilaments while the PB2 (arrow-2) is also forming a spindle. The inset shows a monopolar (arrowhead) and a bipolar (arrow) spindle around a decondensed sperm nucleus and maternal chromosomes, respectively, in a cryopreserved oocyte fixed at 8 hr postinsemination, indicating bypass of the pronuclear formation. Bar = 20 μ m.

Table I. Cytoskeletal Organization and Chromosome Alignment of Mouse Oocytes Cryopreserved at the GV Stage and Fixed After In Vitro Maturation^a

Group	Percentage			
	Normal microfilament pattern	Normal microtubule pattern	Barrel-shaped spindle	Normal chromosome alignment
Control	95 (59/62)	95 (59/62)	85 (53/62)	94 (58/62)
Cryopreservation	95 (61/64)	100 (64/64)	78 (50/64)	86 (55/64)

^a Experiments were repeated three times. Differences between two groups are not statistically significant.

tion (bipolar around egg chromosomes and monopolar around the sperm head) was complete in some cases and egg chromosomes were aligned at the equatorial plate (inset in Fig. 2D). These findings indicate that cryopreservation at the GV stage caused abnormalities in development by two opposite ways (either arrest or abnormal acceleration of development by skipping the pronuclear stage).

Polyploidy

To elucidate the effect of cryopreservation at the GV stage on polyploidy, the numbers of polyspermic and digynic eggs in each group were determined, and these findings are shown in Table II. Neither polyspermy nor digyny was significantly increased by cryopreservation (10 and 3%, respectively) compared with controls (6 and 3%, respectively). The cytoskeletal organization of polyspermic eggs was generally similar to that of diploid pronuclear eggs despite the extra pronucleus/pronuclei. In contrast, digynic eggs dis-

played abnormalities in PB2 formation and in microtubular spindle remnant. Instead of regular PB2, they formed a small round body next to the spindle remnant. The spindle remnant was tiny and abnormal in shape compared to that of diploid eggs fixed at 8 hr postinsemination.

DISCUSSION

Cryopreservation of MII oocytes has been shown to result in partially irreversible spindle damage (3,4,11,12), and it is thought that absence of the spindle at the GV stage may be advantageous to overcome this problem. Indeed, our present results show that, in contrast to MII oocytes, GV-stage oocytes respond to the same slow-cooling and slow-thawing protocol with no significant abnormalities in cytoskeletal organization, spindle morphology, or chromosome alignment. These findings are in agreement with those of Van der Elst *et al.* (15), who also did not find any major abnormalities in morphology of the second meiotic spindle after ultrarapid freezing of GV-stage mouse oocytes. Similarly, cryopreservation of human GV-stage oocytes using a slow-cooling and rapid-thawing protocol spares abnormalities in the resulting second meiotic spindle (16). Collectively, these findings show that cryopreservation at the GV stage may be an alternative to circumvent partially irreversible freezing injury to the spindle. It is, however, noteworthy that, in contrast to the findings of Baka *et al.* (16), a recent study reported significant increase in spindle and chromosome abnormalities after rapid freezing and in vitro maturation of human GV-stage oocytes (17). These contrasting results might be due to differences in cryopreservation protocol used in both studies. Further research is needed to clarify controversial results obtained after cryopreservation of human oocytes.

Table II. Cytoskeleton-Dependent Fertilization Events and Polyploidy in Mouse Oocytes Cryopreserved at the GV Stage and Fixed 8 hr Postinsemination^a

	Percentage	
	Control	Cryopreservation
Sperm penetration (penetrated/inseminated)	89 (66/74)	88 (70/80)
Spindle rotation	98 (65/66)	96 (67/70)
Formation of second polar body	97 (64/66)	94 (66/70)
Formation of pronuclei	100 (66/66)*	81 (57/70)*
Migration of pronuclei	45 (30/66)	37 (21/57)
Polyspermy (polyspermic/fertilized)	6 (4/66)	10 (7/70)
Digyny (digynic/fertilized)	3 (2/66)	3 (2/70)

^a Experiments were repeated four times.

* $P = 0.0007$.

An increased polyploidy rate has been reported previously after fertilization of cryopreserved MII human (5) and mouse (6,7) oocytes. Our previous study revealed that cryopreservation at the MII stage does not increase polyspermy but rather increases digyny (3). Based on the evidence in that study, we suggested that increased digyny is related to partially irreversible spindle damage after cryopreservation at the MII stage rather than primary malfunction of microfilaments. Considering both the absence of significant spindle abnormalities and the low digyny rate in the present study, it is plausible to conclude that bypassing the irreversible spindle damage by performing cryopreservation at the GV stage also avoids increased digyny after cryopreservation.

We have further examined the effect of cryopreservation at the GV stage on the functionality of oocyte cytoskeleton during fertilization by evaluation of cytoskeleton-dependent events. In both the cryopreservation and the control groups, spindle rotation, PB2 formation, and pronuclear migration occurred in a similar fashion, indicating that cryopreservation at the GV stage did not impair the functionality of microfilaments and microtubules during fertilization. However, pronuclear formation was significantly inhibited by cryopreservation. This inhibition must be due to failure rather than delay in this process, because our previous results showed that even in the case of cryopreservation-caused delay in fertilization events, pronuclear formation is completed by 8 hr postinsemination (3). The formation of a microtubular network in cryopreserved eggs that failed to form pronuclei further supports this conclusion because microtubules in the eggs with decondensed sperm head normally appear as cortical asters. Moreover, some of the eggs that failed to form pronuclei already skipped this stage and formed or began to form a mitotic spindle, indicating lack of cell cycle control in these eggs, an aspect deserving further research. The failure of pronuclear formation may be the reason for the decreased cleavage and development rates observed in earlier studies (15,18–20) because eggs with this kind of abnormalities are known to be deficient for development.

In the mouse, microtubule inhibitors prevented normal decondensation of the incorporated sperm nucleus, indicating also microtubule dependence of pronuclear formation (9). Because the failure of pronuclear formation in the present study occurred after decondensation of sperm nucleus, it seems unlikely that a cryopreservation-induced subtle damage to microtubules is the primary underlying cause for inhibition of pronuclear formation. In fact, microtubules appeared to respond

properly to the changes in chromatin pattern even in those cryopreserved oocytes that failed to form pronuclei. We believe that activity of some enzymes and/or synthesis of some molecules involved in pronuclear formation (21,22) was insufficient or perturbed by cryopreservation-associated stresses. It has been well recognized that somatic cell–oocyte interactions are crucial for complete cytoplasmic maturation and, thus, normal development. Removal of cumulus cells before in vitro oocyte maturation leads to reduced cleavage and embryonic development compared with those of cumulus-enclosed oocytes matured in vitro (23,24). Although we used fully cumulus-enclosed oocytes in this study, cumulus cells around some oocytes appeared to be loosened after cryopreservation, indicating some damage and thereby potential compromise from their functional support. This could be responsible for incomplete cytoplasmic maturation with a consequence of failure in pronuclear formation.

In summary, the present study shows that cryopreservation-induced damage to the oocyte cytoskeleton, especially to the microtubular spindle, can be avoided by performing cryopreservation at the GV stage. This, in turn, helps circumvent a possible increase in digyny rate due to damaged spindle. Our results also show that cryopreservation at the GV stage does not adversely affect the ability of oocytes to undergo in vitro maturation and sperm penetration. In addition, our findings demonstrating that cryopreservation at the GV stage significantly inhibits pronuclear formation provide an explanation for the decreased cleavage and development rates reported in the literature after cryopreservation of GV oocytes.

ACKNOWLEDGMENTS

This work was partially supported by research grants from the American Cancer Society, TAP Pharmaceuticals, and the Shriners Hospital for Children.

REFERENCES

1. Magistrini M, Szollosi D: Effects of cold and of isopropyl-N-phenylcarbamate on the second meiotic spindle of mouse oocytes. *Eur J Cell Biol* 1980;22:699–707
2. Aman RR, Parks JE: Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. *Biol Reprod* 1994;50:103–110
3. Eroglu A, Toth TL, Toner M: Alterations of the cytoskeleton and polyploidy induced by cryopreservation of metaphase II mouse oocytes. *Fertil Steril* 1998;69:944–957

4. Vincent C, Garnier V, Heyman Y, Renard JP: Solvent effects on cytoskeletal organization and in-vivo survival after freezing of rabbit oocytes. *J Reprod Fert* 1989;87:809-820
5. Al-Hasani S, Diedrich K, Van der Ven H, Reinecke A, Hartje M, Krebs D: Cryopreservation of human oocytes. *Hum Reprod* 1987;2:695-700
6. Glenister PH, Wood MJ, Kirby C, Whittingham DG: Incidence of chromosome anomalies in first-cleavage mouse embryos obtained from frozen-thawed oocytes fertilized in vitro. *Gamete Res* 1987;16:205-216
7. Carroll J, Warnes GM, Matthews CD: Increase in digyny explains polyploidy after in-vitro fertilization of frozen-thawed mouse oocytes. *J Reprod Fert* 1989;85:489-494
8. Maro B, Johnson MH, Webb M, Flach G: Mechanism of polar body formation in the mouse oocytes: an interaction between the chromosomes, the cytoskeleton and the plasma membrane. *J Embryol Exp Morphol* 1986;92:11-32
9. Schatten G, Simerly C, Schatten H: Microtubule configurations during fertilization, mitosis, and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. *Proc Natl Acad Sci USA* 1985;82:4152-4156
10. Sathananthan AH, Trounson A, Freeman L, Brady T: The effects of cooling human oocytes. *Hum Reprod* 1988;3:968-977
11. Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J: Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocytes. *Fertil Steril* 1990;54:102-108
12. Van der Elst J, Van den Abbeel E, Jacobs R, Wisse E, Van Steirteghem A: Effect of 1,2-propanediol and dimethylsulphoxide on the meiotic spindle of the mouse oocyte. *Hum Reprod* 1988;3:960-967
13. Mattson BA, Albertini DF: Oogenesis: Chromatin and microtubule dynamics during meiotic prophase. *Mol Reprod Dev* 1990;25:374-383
14. Toth TL, Jones HW, Baka SG, Muasher S, Veeck LL, Lanzendorf S: Fertilization and in vitro development of cryopreserved human prophase I oocytes. *Fertil Steril* 1995; 61:891-894
15. Van der Elst JC, Nerinckx SS, Van Steirteghem AC: In vitro maturation of mouse germinal vesicle-stage oocytes following cooling, exposure to cryoprotectants and ultrarapid freezing: Limited effect on the morphology of the second meiotic spindle. *Hum Reprod* 1992;7:1140-1146
16. Baka SG, Toth TL, Veeck LL, Jones Jr HW, Muasher SJ, Lanzendorf SE: Evaluation of the spindle apparatus of in vitro matured human oocytes following cryopreservation. *Hum Reprod* 1995;7:1816-1820
17. Park SE, Lee KA, Son WY, Ko JJ, Lee SH, Cha KY: Chromosome and spindle configurations of human oocytes matured in vitro after cryopreservation at the germinal vesicle stage. *Fertil Steril* 1997;68:920-926
18. Schroeder AC, Champlin AK, Mobraaten LE, Eppig JJ: Developmental capacity of mouse oocytes cryopreserved before and after maturation in vitro. *J Reprod Fert* 1990;89:43-50
19. Van der Elst JC, Nerinckx SS, Van Steirteghem AC: Slow and ultrarapid freezing of fully grown germinal vesicle-stage mouse oocytes: Optimization of survival rate outweighed by defective blastocyst formation. *J Assist Reprod Genet* 1993;10:202-212
20. Van Blerkom J and Davis PW: Cytogenetic, cellular, and developmental consequences of cryopreservation of immature and mature mouse and human oocytes. *Microsc Res Tech* 1994;27:165-193
21. Ding J, Moor RM, Foxcroft GR: Effects of protein synthesis on maturation, sperm penetration, and pronuclear development in porcine oocytes. *Mol Reprod Dev* 1992;33:59-66
22. Szollosi MS, Kubiak JZ, Debey P, De Pennart H, Szollosi D, Maro B: Inhibition of protein kinases by 6-dimethylaminopurine accelerates the transition to interphase in activated mouse oocytes. *J Cell Sci* 1993;104:861-872
23. Schroeder AC, Eppig JJ: The developmental capacity of mouse oocytes that matured spontaneously in vitro is normal. *Dev Biol* 1984;102:493-497
24. Schramm RD, Bavister BD: FSH-priming of rhesus monkeys enhances meiotic and developmental competence of oocytes in vitro. *Biol Reprod* 1994;52:904-912