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Meiotic spindle dynamics in human oocytes following slow-cooling cryopreservation

J.J. Bromfield[1], G. Coticchio[2], K. Hutt[1], R. Sciajno[2], A. Borini[2], and D.F. Albertini[1,3]

[1]Department of Molecular and Integrative Physiology, Centre for Reproductive Sciences, University of Kansas Medical Centre, Kansas City, KS, USA [2]Tecnobios Procreazione, Centre for Reproductive Health, Bologna, Italy

[3]Correspondence address. E-mail: dalbertini@kumc.edu

BACKGROUND: The demand for cryopreservation of human oocytes is increasing in assisted reproduction clinics and yet remains an experimental procedure. Surprisingly, little is known about the effects of cryopreservation on spindle–chromosome interactions and the recovery of meiotic spindle functionality. The goal of these studies was to evaluate the process of meiotic spindle reassembly and chromosome alignment in cryopreserved human metaphase II oocytes.

METHODS: Unfrozen control oocytes were compared with frozen oocytes fixed at 0, 1, 2 and 3 h after thawing. Oocytes were analysed by confocal microscopy and subjected to 3-dimensional image analysis to evaluate spindle integrity.

RESULTS: Freezing resulted in a loss of spindle bipolarity and chromosome alignment. One hour following thawing, most oocytes recovered spindle bipolarity and equatorial chromosomal alignment. However, between 2 and 3 h, a progressive loss of chromosome alignment was observed. Further analysis revealed a positive correlation between spindle length and number of displaced chromosomes following freezing. This time-dependent redistribution of chromosomes involved outward displacement from the equatorial plate and retention at the surface of the meiotic spindle.

CONCLUSIONS: Spindle disassembly incurred by cryopreservation is rapidly reversed and is coordinated with chromosome alignment within 1 h but is not sustained at later times.

Key words: chromosome alignment / cryopreservation / meiotic spindle / microtubule / oocyte

Introduction

The high incidence of aneuploidy evident in human embryos produced by natural or artificial conception has been linked to defects in oocyte meiotic and/or embryo mitotic spindles (Pellestor, 1991; Mailhes *et al*., 1998; Hassold and Hunt, 2001; Chatzimeletiou *et al*., 2005; Kuliev *et al*., 2005) Many fundamental questions remain as to the causes of aneuploidy with regard to the dynamic nature of microtubule (MT)–chromosome interactions in the human oocyte or embryo especially in the context of developing safe and efficacious protocols for human oocyte cryopreservation. Surprisingly, the effects of cooling on spindle MT dynamics in somatic and germ cells figured prominently in development of the dynamic equilibrium model of MT assembly through the classical studies of Inoue and others using polarization microscopy (Inoue, 1953; Sato *et al*., 1975). This form of microscopy has entered the realm of clinical assisted reproductive techniques (ARTs) and has become useful in assessments of oocyte quality for human embryo production with and without cryopreservation (Keefe *et al*., 2003; Coticchio *et al*., 2009). To gain further insight into the biomechanical properties of spindle MTs and chromosomes in human oocytes, high resolution confocal microscopy has been used to define structural properties of human oocyte meiotic spindles related to aneuploidy during advancing maternal age (Battaglia *et al*., 1996; Volarcik *et al*., 1998). To date, however, few studies have employed the analytical capabilities of confocal microscopy in conjunction with oocyte cryopreservation protocols, where freezing and thawing could be used as an experimental approach towards understanding the biophysical properties of the human meiotic spindle (Coticchio *et al*., 2006; De Santis *et al*., 2007).

The rapid recovery of form birefringence during spindle reassembly after chilling has been reported using polarization microscopy but specific parameters of chromosome disposition and spindle integrity are more difficult to resolve with the Polscope (Keefe *et al*., 2003; Coticchio *et al*., 2009). Moreover, although most clinics use a recovery

time of several hours prior to ICSI or IVF based on birefringence detection, the exact series of events following spindle recovery in cryopreserved human oocytes have yet to be studied. Thus, the purpose of this study was to monitor both the time course and chromosome–MT characteristics of human meiotic spindles from zero to 3 h after thawing using confocal microscopy, high resolution optical sectioning, and 3-dimensional image analysis. Our results indicate that although spindle reassembly and chromosome alignment are achieved within 1 h after thawing, chromosomes become repositioned by 3 h and likely contribute to the heightened incidence of spontaneous miscarriages observed after fertilization of cryopreserved human oocytes.

Materials and Methods

Oocyte collection and patient approval

This study was approved by the Public Health Agency of the Italian government, National Health Institute and the Institutional Review Boards of the participating clinics. Oocytes were obtained from consenting couples undergoing ART for male and unexplained infertility or, in one case, endometriosis. Controlled ovarian stimulation was induced with long protocols using GnRH agonist and recombinant FSH, according to the standard clinical procedures routinely employed by the participating clinics (Borini *et al.*, 2007). Ten thousand IU of hCG were administered 36 h prior to oocyte collection. After retrieval and before cryopreservation, oocytes were cultured in IVF media (Cook IVF, Brisbane, Australia, or Sage IVF Inc., Trumbull, CT, USA) for 2–3 h. During that period of time, complete removal of cumulus and corona cells was performed using hyaluronidase (40 IU/ml), and pipetting. Only mature oocytes demonstrating spherical, regular boundaries, and an extruded first polar body [thus presumably at the metaphase II (MII) stage] were allocated to the study. The number of participating patients, total number of oocytes analysed, oocytes per patient and patient age are summarized in Table I. No significant difference in the mean age of patients between groups was detected and it should be noted that although the total age range of patients included in this study is 26–43 years of age, only three of the 32 patients lie outside of the age range of 30–40 years. The eight oocytes derived from these three patients are included in the present analysis because this data did not vary from the trends reported for each of the time groups examined.

Oocyte cryopreservation, storage, thawing and culture

Oocytes were cryopreserved using a slow cooling method. All cryopreservation solutions were prepared using Dulbecco's phosphate-buffered solution (PBS) (Gibco, Life Technologies Ltd, Paisley, Scotland) and a Plasma Protein Supplement (PPS) (10 mg/ml, final concentration) (BAXTER AG, Vienna, Austria).

The equilibration solution contained 1.5 mol/l PrOH + 20% PPS in PBS and the loading solution consisted of 1.5 mol/l PrOH + 0.3 mol/l sucrose + 20% PPS in PBS, as described by Fabbri *et al.* (2001). The thawing solutions contained a gradually decreasing concentration of PrOH and a constant 0.3 mol/l sucrose concentration. They were prepared as follows: (i) 1.0 mol/l PrOH + 0.3 mol/l sucrose + 20% PPS; (ii) 0.5 mol/l PrOH + 0.3 mol/l sucrose + 20% PPS; (iii) 0.3 mol/l sucrose + 20% PPS.

Cumulus-free oocytes were incubated in equilibration solution for 10 min at room temperature (RT) and transferred to loading solution for 5 min. Oocytes were loaded in plastic straws (Paillettes Crystal 133 mm; Cryo Bio System, France) individually or in small groups (maximum of three oocytes per straw). The temperature was lowered through an automated Kryo 10 series III biological freezer (Planer Kryo 10/1,7 GB) from 20°C to −8°C at a rate of 2°C/min. Manual seeding was performed at −8°C and this temperature was maintained for 10 min to allow for uniform ice propagation. Temperature was then decreased to −30°C at a rate of 0.3°C/min, rapidly brought to −150°C at a rate of 50°C/min and straws were plunged into liquid nitrogen at −196°C and stored for later use.

Thawing was carried out at RT. Plastic straws were held in air at RT for 30 s and transferred to a water bath at 30°C for 40 s. Stepwise cryoprotective agent dilution was achieved by transferring oocytes from thawing solution (i) 5 min to solution (ii) for 5 min and finally into solution (iii) for 10 min before final dilution in PBS + 20% PPS for 20 min (10 min at RT and 10 min at 37°C). During post-thaw culture, oocytes were incubated in glucose-free cleavage medium (Cook IVF, or Sage IVF Inc.).

Oocyte fixation and immuno-staining

Oocytes were fixed either prior to freezing (unfrozen; $n = 24$), immediately after thawing and rehydration (time 0; $n = 24$) or following 1 h ($n = 39$), 2 h ($n = 19$) or 3 h ($n = 24$) post thaw culture. Oocytes were fixed in MT stabilizing buffer (100 mM PIPES, 5 mM $MgCl_2$, 2.5 mM EGTA, 2% formaldehyde, 0.1% Triton-X-100, 1 mM taxol, 10 U/ml aprotinin and 50% deuterium oxide) for 30 min at 35°C and stored in wash solution (0.2% sodium azide, 2% normal goat serum, 1% BSA, 0.1% glycine and 0.1% triton X-100 in PBS) as described previously (Combelles *et al.*, 2002). Samples were incubated overnight in the presence of an antibody cocktail containing both mouse anti-α-tubulin and mouse anti-β-tubulin or mouse anti-acetylated tubulin alone at 4°C (all 1:100, Sigma-Aldrich, St Louis, MO, USA). Antibody detection was performed using a goat anti-mouse-alexa488 (1:800, Molecular Probes, Carlsbad, CA, USA) secondary antibody in combination with Phalloidin-Alex 568 (1:100,

Table I Number of donors, oocytes and age of oocytes analysed for total tubulin

Treatment	Number of donors	Number of oocytes at MII	Number of MII oocytes from each patient	Number of oocytes at telophase (% total)	Number of activated oocytes (% total)	Age (range)
Unfrozen	6	22	1, 2, 3, 3, 4, 9	1 (4.2)	1 (4.2)	37.5 ± 2.0 (30–43)
T0	8	22	1, 1, 1, 2, 3, 3, 5, 6	2 (8.3)	0 (0)	36.8 ± 2.1 (35–40)
T1	5	35	3, 6, 7, 7, 12	1 (2.6)	3 (7.7)	34.8 ± 1.9 (33–38)
T2	6	19	2, 3, 3, 3, 3, 5	0 (0)	0 (0)	34.0 ± 5.5 (26–40)
T3	7	22	1, 2, 2, 3, 4, 5, 5	2 (8.3)	0 (0)	35.1 ± 1.5 (29–40)

Age is represented as mean ± s.e.m years. Only oocytes analysed for total tubulin are included. There was no significant difference of patient ages between any groups.

Molecular Probes) for the detection of total actin and 1 μg/ml of Hoechst 33258 (Molecular Probes) at 37°C with gentle agitation. Samples were washed three times in wash solution for a total of 45 min at 37°C. Samples were mounted in medium containing 50% glycerol/PBS containing 25 mg/ml sodium azide and 1 μg/ml of Hoechst 33258 using wax cushions to avoid compression of samples.

Image acquisition and analysis

Samples were analysed on a Zeiss LSM 5 Pascal confocal microscope using a 63× C-Plan-Apochromat objective (na = 1.4), KrArg (405 488 nm excitation) and HeNe (543 nm) lasers for collection of complete three channel z-stacks through the entire spindle of each oocyte. Optical sections were collected at 0.6 μm intervals and reconstructed as 3-dimensional projections for assignment of specific spindle and chromosomal properties (shape, length and polar constriction). Each oocyte was further characterized based on whether all chromosomes were aligned at the metaphase plate or defined as misaligned if one or more chromosomes were dislocated from the metaphase plate.

Spindle pole to pole length was measured using Zeiss Pascal software in X, Y or Z planes so that measurements could be calculated in all oocytes regardless of spindle orientation. Spindle MT volume was assessed using Metamorph v7.10 (Universal Imaging Corporation, Buckinghamshire, UK) from full reconstructions of Z plane data sets for tubulin staining in each spindle after calibration for magnification and thresholding against cytoplasm.

Statistical analysis

Statistical analysis was carried out on pooled samples comparing unfrozen control samples to samples from T0, T1, T2 and T3 using a chi squared test when comparing percentages. A Pearson correlation test was performed to determine the correlation between chromosomal displacement and spindle length. All other data was analysed using a one-way ANOVA followed by a Sidak *post-hoc* test. A *P*-value <0.05 was deemed as statistically significant.

Results

Oocyte assessment of meiotic stage

A total of 130 oocytes from 32 patients were fixed and stained for total tubulin, DNA and actin (Table I). Of these 130 oocytes a total of 10 oocytes were excluded from analysis because they exhibited parthenogenetic activation as evidenced by the restoration of cytoplasmic MTs, chromatin decondensation and polar bodies (see Combelles *et al.*, 2003). The incidence of activation was not attributable to cryopreservation as comparable numbers were present in the unfrozen control group. An additional 21 frozen-thawed oocytes were labelled for acetylated tubulin to determine if MT stabilization occurred during thawing and recovery.

Spindle bipolarity and chromosomal alignment as independent measures of spindle integrity

Following confocal microscopy, 3-dimensional reconstructions of each spindle were used to classify bipolar versus disarranged spindles at the various time points following oocyte thawing. Spindle bipolarity was significantly affected by cryopreservation and showed striking dynamics upon thawing. Unfrozen control oocytes (100.0%) displayed bipolar spindles with constriction evident at both poles. Following cryopreservation, there was a significant reduction of oocytes with bipolar spindles directly following thawing (T0; 59.1% bipolar), although after 1 h of culture (T1) 85.7% of oocytes regained bipolar spindles. Oocytes cultured for 2 (T2) or 3 h (T3) following thawing displayed 73.7 and 72.7% bipolar spindles, respectively (Table II).

In unfrozen control samples, only 59.1% of oocytes displayed all chromosomes aligned along the metaphase plate regardless of spindle bipolarity. Following cryopreservation, a significant reduction in the incidence of oocytes displaying chromosome alignment along the metaphase plate was observed directly following thawing (T0; 13.6%), whereas after 1 h of culture (T1) 77.1% of oocytes demonstrated complete metaphase plate alignment. Oocytes cultured for 2 (T2) or 3 h (T3) after thawing maintained chromosome alignment to an extent comparable to unfrozen control oocytes (42.1 and 45.5%, respectively) (Table II). It is interesting to note that when chromosome alignment in frozen thawed oocytes was assessed relative to patient age (<35 versus >36 years), only 11.1% of oocytes from older patients maintain chromosome alignment compared with 70.0% of oocytes from younger patients at T2 ($P < 0.05$). A similar trend is evident at T3 where only 41.7% of oocytes from older patients maintain chromosome alignment compared with 60.0% in oocytes from younger patients.

Table II Spindle bipolarity and chromosomal alignment after thawing and *in vitro* culture

Treatment (patients)	Spindle bipolarity (%)		Chromosomal alignment (%)	
	Bipolar	Disarranged	Aligned	Non-aligned
Unfrozen (6)	22/22 (100.0)	0/22 (0.0)	13/22 (59.1)	9/22 (40.9)
T0 (8)	13/22 (59.1)*	9/22 (40.9)	3/22 (13.6)	19/22 (86.4)*
T1 (5)	30/35 (85.7)	5/35 (14.3)	27/35 (77.1)	8/35 (22.9)
T2 (5)	14/19 (73.7)*	5/19 (26.3)	8/19 (42.1)	11/19 (57.9)
T3 (7)	16/22 (72.7)*	6/22 (27.3)	10/22 (45.5)	12/22 (54.5)

Chi squared test between unfrozen controls and frozen samples comparing oocytes with bipolar spindles or aligned chromosomes.
*$P < 0.05$.

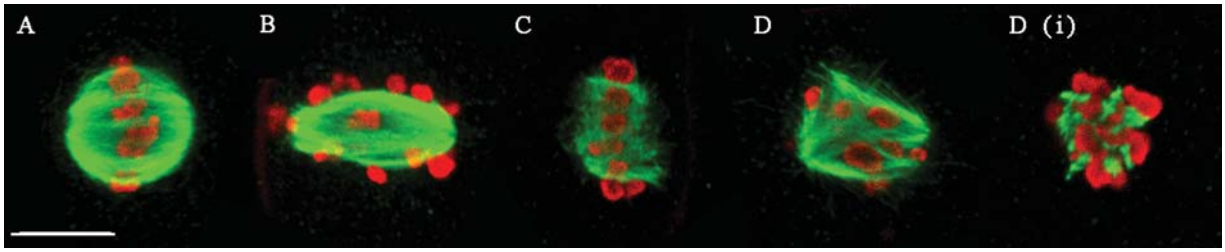


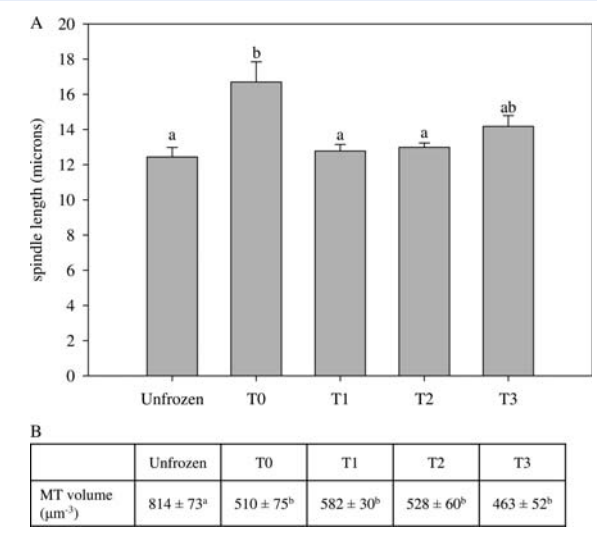
Figure 1 Representative confocal images of meiotic machinery following cryopreservation.

Meiotic spindles were classified as: (**A**) bipolar spindle with chromosomes aligned along the equatorial plate; (**B**) bipolar spindle with non-aligned, scattered chromosomes; (**C**) disarranged spindle with aligned chromosomes or (**D**) disarranged spindle with non-aligned, scattered chromosomes; (**Di**) is representative of a spindle allocated to class D where the spindle is absent or a few MT fibres persist. Note the lack of MT pole focusing and kinetochore bundling in D. Total tubulin is represented by green and DNA by red. Scale bar represents 10 μm.

Spindle bipolarity and chromosomal alignment as co-operative measures of spindle integrity

The meiotic spindle apparatus was categorized for each oocyte as previously described (De Santis *et al*., 2007). Briefly, oocytes were categorized as either bipolar spindle with all chromosomes aligned (Fig. 1A), bipolar spindle with chromosomes displaced from the equatorial plate (Fig. 1B), or multipolar/monopolar spindles in which chromosomes were aligned (Fig. 1C) or scattered (Fig. 1D).

Most unfrozen control oocytes (59.1%) contained bipolar spindles with aligned chromosomes. Oocytes analysed directly after freezing (T0) displayed no bipolar spindles with aligned chromosomes (0.0%), although any bipolar spindles contained scattered chromosomes (59.1%). By 1 h post thaw (T1) a significant majority of oocytes (71.4%) recovered spindle bipolarity and aligned chromosomes reinforcing trends described above as independent measure of a 1 h recovery. Notably, one (T2) or two (T3) additional hours of culture revealed a failure to maintain spindle bipolarity and chromosome alignment relative to oocytes cultured for only 1 h (31.6 and 22.7%, respectively). These oocytes not only exhibited loss of spindle bipolarity, but also lacked pole constriction and kinetochore bundling. These alterations were unique to frozen oocytes, and were maintained throughout the 3 h culture period (Table III) and differed from variations observed in fresh oocytes that were not subjected to cryopreservation.



B

	Unfrozen	T0	T1	T2	T3
MT volume (μm^{-3})	814 ± 73^{a}	510 ± 75^{b}	582 ± 30^{b}	528 ± 60^{b}	463 ± 52^{b}

Figure 2 Spindle pole to pole length as determined by post-thaw culture period.

Spindle pole to pole length was measured in oocytes containing bipolar spindles in unfrozen controls and at 0 (T0), 1 (T1), 2 (T2) and 3 (T3) hours post-thaw culture of frozen oocytes. Spindle MT volume is shown below the *x*-axis. Bars represent the mean length in microns ± s.e.m. MT volumes are shown in μm^3. Different superscripts represent statistically significant differences following a one-way ANOVA, $P < 0.05$.

Progressive/regressive changes to spindle morphology after thawing

Image analysis was used to further characterize the interactions between chromosome alignment and spindle dynamics. Pole to pole length of bipolar spindles were assayed in relation to post-thaw time independent of chromosome alignment. Unfrozen control oocytes displayed an average spindle length of 12.4 ± 0.5 μm, comparable to reported spindle lengths in human oocytes by others (Battaglia *et al*., 1996; Combelles *et al*., 2002, 2003). Surprisingly, pole to pole length was significantly increased in bipolar spindles directly after thawing (T0) compared with unfrozen controls or oocytes cultured for 1 or 2 h post-thaw (25.5, 23.5 and 22.2%, respectively; $P < 0.005$) (Fig. 2A). This finding suggests that spindle elongation is a direct consequence of freezing and thawing. It is important to note that the majority of oocytes with elongated bipolar spindles at T0 display non-aligned chromosomes. The question of whether spindle elongation is the result of enhanced MT assembly was addressed by measuring total tubulin immunolabeling in individual oocytes (Fig. 2B). Unfrozen oocytes contain a mean MT volume of 814 ± 73 μm^3, however, oocytes subjected to freeze/thawing displayed significantly reduced MT volumes between T0 and T3 (509 ± 75 (T0), 582 ± 30 (T1), 528 ± 60 (T2) and 463 ± 52 (T3) μm^3). This finding suggests that frozen/thawed oocytes exhibit a significantly decreased absolute mass of spindle tubulin when compared with unfrozen MII oocytes (Fig. 2B). These results further emphasize the

Table III Frequency of meiotic machinery morphology following cryopreservation

Treatment (patients)	Bipolar spindle, aligned chromosomes (%)	Bipolar spindle, non-aligned chromosomes (%)	Disarranged spindle, aligned chromosomes (%)	Disarranged spindle, non-aligned chromosomes (%)
Unfrozen (6)	13/22 (59.1)	9/22 (40.9)	0/22 (0.0)	0/22 (0.0)
T0 (8)	0/22 (0)*	13/22 (59.1)	3/22 (13.6)	6/22 (27.3)
T1 (5)	25/35 (71.4)	5/35 (14.3)	2/35 (5.7)	3/35 (8.6)
T2 (5)	6/19 (31.6)‡	8/19 (42.1)	2/19 (10.5)	3/19 (15.8)
T3 (7)	5/22 (22.7)*	11/22 (50.0)	6/22 (27.3)	0/22 (0.0)

Chi squared test between unfrozen controls and frozen samples comparing oocytes with bipolar spindles and aligned chromosomes.
*$P < 0.05$, ‡$P < 0.08$.

fact that despite increased length, as for example at T1, all oocytes subjected to freeze/thaw regain normal spindle parameters but with decreased MT volume. This prompted the question of whether changes in spindle length were related to the state of chromosome organization.

Chromosome alignment impacts spindle length

As heterogeneity between oocytes would be expected after freeze/thaw given potential variance in patient age and/or responsiveness to gonadotrophins, we next explored the relationship between spindle length and chromosome disposition independent of time post thaw. For oocytes lacking bipolarity, the longest distance between MT ends running perpendicular to the metaphase plate was recorded. Oocytes within bipolar (chromosome aligned or not) or apolar (aligned) classes yielded consistent measurements with little variation (Fig. 3). Oocytes of bipolar spindle aligned class were shorter in length (12.5 ± 0.3 μm) compared with oocytes with bipolar spindles and scattered chromosomes (14.5 ± 0.5 μm; $P < 0.001$). Oocytes bearing disarranged spindle/aligned chromosomes exhibited even smaller pole–pole lengths (10.3 ± 0.6 μm; $P < 0.05$ compared with both bipolar spindle categories). Oocytes containing a disarranged spindle and misaligned chromosomes were excluded from this analysis (only 41% of oocytes) due to difficulties in obtaining pole–pole distances. Because the number of displaced chromosomes varied between samples, we next examined spindle lengths as a function of how many chromosomes failed to remain in the metaphase plate.

The number of displaced chromosomes on bipolar spindles was determined from 3D reconstructions for individual oocytes. A positive correlation between spindle length and the number of displaced chromosomes was observed ($P < 0.001$) (Fig. 4). Interestingly, displacements of 1–3 chromosomes are associated with little overall change in spindle length whereas >3 displaced chromosomes results in elongation to varying degrees (range of 11–27 μm). Representative examples of these are illustrated in Fig. 5. Note that in some cases, a single displaced chromosome is associated with spindle elongation (Fig. 5C) to the same extent as spindles with extensive chromosomal dispersion (Fig. 5H). During the analysis of chromosome displacement, it was noted that significant variations in the structure, size and positioning of chromosomes appeared to be related to spindle length.

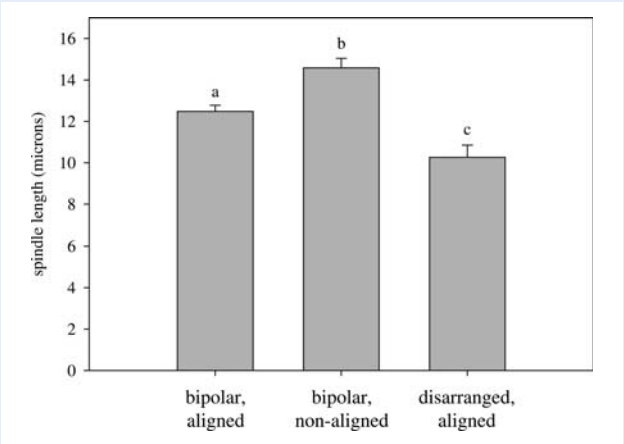


Figure 3 Spindle length as determined by spindle morphological classification.

Spindle pole to pole length measurements were made as described in the text as a function of spindle subclasses excepting those with disarranged spindles and scattered chromosomes. Bars represent the mean length in microns ± s.e.m. Different superscripts represent statistically significant differences following a one-way ANOVA, $P < 0.05$.

As the above data indicates (Fig. 5), bipolar MII spindles from fresh or frozen oocytes exhibit a wide range of chromosome displacement patterns from equatorial plate alignment to widespread scattering. Strikingly, in these and past studies, it appears that human chromosomes retain an avidity for the spindle apparatus despite conditions that predispose oocytes to meiotic aneuploidy (maturation aging or, as here, freezing). It was therefore of interest to more closely inspect the nature of chromosome–MT interactions within various bipolar spindles using reconstructions that would permit chromosome position to be analysed in X, Y and Z planes (Fig. 6). In cases of complete chromosome alignment (Fig. 6A), two concentric rings of chromosomes are evident that occupy indentations in the compact spindle. In cases where at least one chromosome is segregated away from the metaphase plate, the remaining chromosomes retain a loose association with MTs whether they are located within the spindle centre or at its surface (Fig. 6B). Finally, many instances were observed where elongate spindles are covered by displaced chromosomes (Fig. 6C). In such cases, image rotations confirm that

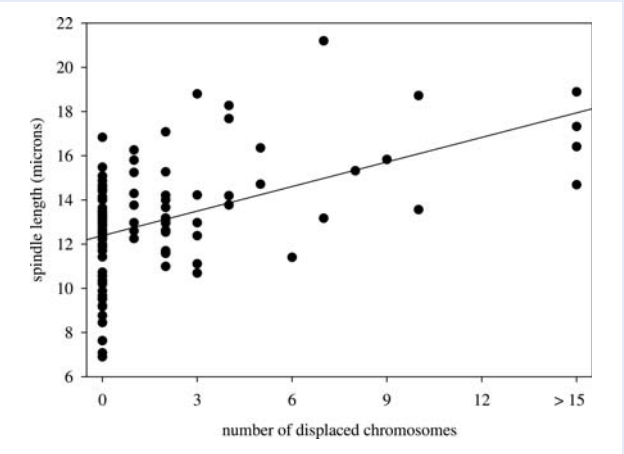


Figure 4 Correlation between the number of displaced chromosomes and spindle length.

Scatter plot showing a positive correlation between the number of dispersed chromosomes and the pole to pole length of individual bipolar spindles. Each dot represents one spindle including those with no displaced chromosomes. Pearson correlation reveals a positive correlation of 0.499 at $P < 0.001$.

chromosomes at the surface retain a tenuous and superficial relationship on the lateral aspects of MTs; not uncommonly, the core chromosomes also seem to be tenuously bound. Based on their number and relative size, it appears that displaced chromosomes represent sister chromatid pairs that have not disjoined implying that the trend towards elongation is not due to precocious activation of the cell cycle or anaphase onset. Thus, human oocytes exhibit a range of chromosome interactions with spindle MTs that are associated with predictable changes in spindle organization.

Alpha-tubulin acetylation is absent in frozen-thawed oocytes

Although previous studies on human oocytes have indicated that meiotic spindles lack acetylation of the tubulin alpha subunit, a MT stabilizing post-translational modification, we studied this possibility to ascertain whether any cold stable MTs were present following freeze/thaw. In addition to the 130 oocytes analysed for total tubulin, 21 frozen-thawed oocytes were probed for tubulin acetylation and although the majority of oocytes contained acetylated MTs within polar bodies and *trans*-zonal projections, no staining was observed in association with the meiotic spindle or chromosomes (data not shown). Thus, oocytes fixed directly after thawing do not show evidence of cold resistant MTs.

Discussion

ARTs can contribute to a decrease in oocyte quality as a direct result of aneuploidy or indirectly due to failure in pre- or post-implantation development. Cryopreservation of human oocytes has emerged as a popular form of ART especially since optimizing this technique holds promise as an approach to fertility preservation. Although various protocols such as slow freeze and vitrification for oocyte cryopreservation are being intensely investigated (Fabbri *et al*., 2001; Boiso *et al*., 2002; Rienzi *et al*., 2004; Kuwayama *et al*., 2005; Coticchio *et al*., 2006; Stachecki *et al*., 2006; Bianchi *et al*., 2007; De Santis *et al*., 2007; Cobo *et al*., 2008), detailed studies on the kinetics of meiotic spindle recovery following cryo-storage are lacking. In light of this, using high resolution confocal microscopy we document the dynamics of the meiotic spindle reassembly in human oocytes with particular attention being paid to the optimal recovery time following thawing of oocytes subjected to a slow-freeze protocol.

Using polarized light microscopy it has been previously established that oocyte cooling leads to the depolymerisation of MTs and a time dependent re-polymerization resulting in the formation of a bipolar spindle in human oocytes by 3 h in culture at 37°C (Pickering *et al*., 1990; Rienzi *et al*., 2004; Bianchi *et al*., 2005). We show here that cryopreserved oocytes immediately following thawing exhibit a range of spindle and chromosome abnormalities that are not observed in unfrozen control oocytes from young or older women. Specifically and importantly, recovery of a bipolar spindle with aligned chromosomes occurs within 1 h of thawing in the majority of oocytes examined. Maintaining oocytes for longer periods of time post thaw results in progressive loss of bipolar spindle structure coincident with chromosome displacement. These findings have direct bearing on the mechanisms at play in establishing and maintaining meiotic spindle integrity after cryopreservation or other ARTs and illustrate the importance of subjecting oocytes to IVF or ICSI sooner than is customarily practiced in ART clinics today.

This work sheds new light on the mechanics of meiotic spindle integrity in human oocytes by taking advantage of the regeneration of this vital structure following cold-induced MT depolymerisation. Although certain trends are reported with respect to the establishment of spindle bipolarity and chromosome alignment, it should be cautioned that there is sufficient variability in these data to suggest that not all oocytes from a single patient or between patients may respond identically reinforcing the notion that human oocytes are heterogeneous in nature. Despite this confounding principle, there was striking consistency in the patterns of spindle recovery detected by a systematic analysis of 120 oocytes from 31 patients.

Our study complements those of others suggesting the dynamic nature of the meiotic spindle in its ability to recover from cooling or cryopreservation (Wang *et al*., 2001; Chen *et al*., 2004). However, when considering the extent of recovery of oocytes in terms of both spindle bipolarity and chromosomal alignment, distinct patterns of meiotic spindle reassembly were found that follow a time-dependent course. Despite the prevalence of disordered spindles with non-aligned chromosomes just following thawing (T0), the appearance of bipolar spindles with aligned chromosomes was dramatic and significant evidencing an active process of spindle repair and chromosome alignment within 1 h. Our work conflicts with other previously reported findings regarding the fate of meiotic spindles with prolonged times in culture. Specifically, a significant fraction of oocytes undergo progressive elongation of the spindle that is accompanied by the displacement of chromosomes from the equatorial plate (Fig. 1C, D). The most immediate implication of this finding is that activation of such oocytes would result in an aberrant anaphase with chromosome non-disjunction and hence an aneuploid constitution at the time of fertilization. Secondarily, the fact that cryopreserved oocytes tend to retain the meiotic spindle in a cortically-affixed configuration indicates that an adequate fertilization

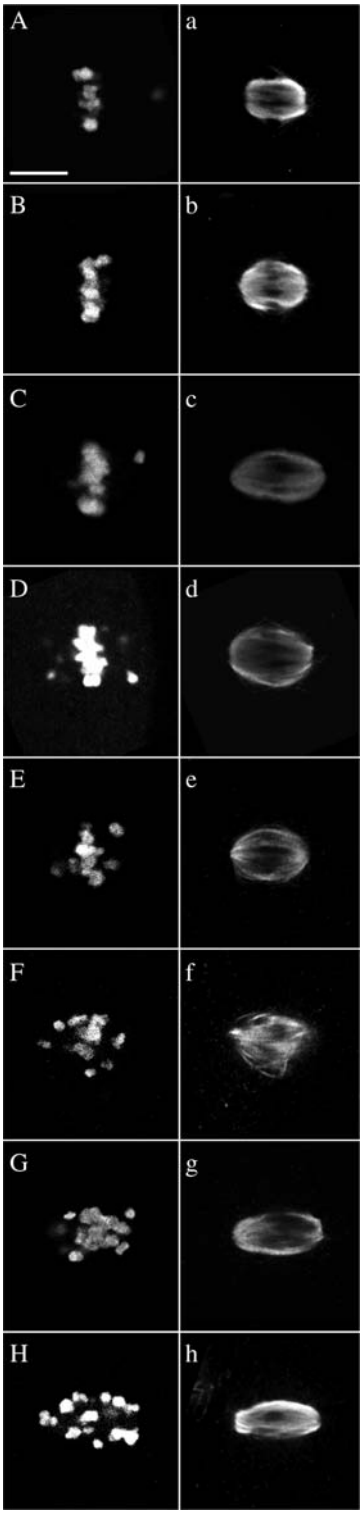


Figure 5 Representative confocal images showing the relationship between chromosome dispersion and spindle length.

Confocal images depicting bipolar spindles (left) with varying degrees of chromosomal displacement (right). A typical bipolar spindle (**a**) with aligned chromosomes (**A**) has a relatively well conserved length. As the number of displaced chromosomes increases (top to bottom), the length of the spindle increases to a point where the majority of chromosomes are displaced and yet spindle bipolarity is maintained (shown in **H, h**). Total tubulin is shown in lower case panels and DNA shown in capitalized panels. Scale bar represents 10 μm.

stimulus provided during IVF or ICSI is unlikely to limit either oocyte activation or subsequent development. This may explain, at least in part, the rather high degree of spontaneous miscarriage rate that is known to occur in some cases during pregnancies generated with cryopreserved MII human oocytes (Levi Setti *et al.*, 2006).

A further question pertains to the causes that underlie such characteristic changes in spindle structure and chromosome positioning. As noted, there is a clear relationship between the number of displaced chromosomes and the overall length of the meiotic spindle (Fig. 5). This time-dependent transformation in human meiotic spindle structure may be a unique consequence to cryopreservation. For example, several studies have called attention to defects in chromosome alignment as a function of maternal age and generally these variations, referred to as defects in the process of chromosome congression, are characterized by wide-ranging degrees of chromosome displacement. Notably, little change in overall spindle length has been reported. Strikingly, the present studies illustrate up to a 14% increase in overall spindle length compared with unfrozen controls or recovered bipolar spindles with no loss of spindle bipolarity or focussing of the spindle poles. This suggests that as oocytes age in culture following cryopreservation, there is a shift in the forces that maintain chromosomes in a metaphase configuration. Whether this change is due to alterations in MT dynamics or the components used to anchor the chromosomes to the spindle remains to be resolved. At the very least, these observations and the experimental approach used herein show that further insights into the mechanics and function of the human meiotic spindle may be obtained from analysis of cryopreserved oocytes at various times following thawing.

Mammalian oocytes are endowed with the machinery that will support the completion of meiosis and the mitotic cell cycles of the early conceptus (Chatzimeletiou *et al.*, 2005). This endowment derives from both the changes in oocyte cytoplasm that occur during the growth and maturative stages of oogenesis within the confines of the ovarian follicle. It is important to ask then what aspects of this endowment are compromised in the course of oocyte cryopreservation that might be responsible for the specific and transient recovery of the spindle that is reported here. As mentioned above, the gradual elongation of the spindle and the parallel displacement of chromosomes from the equatorial plate are suggestive of a heightened propensity for aneuploidy that should be prevented at all costs. We suggest that several factors may be at play that would be required to maintain the integrity of the meiotic spindle following cryopreservation.

Mitotic and meiotic spindles require consumption of energy in the form of GTP and ATP for both their assembly and in the maintenance of bipolarity. Since the energy resources used by the oocyte and early embryo are in large measure drawn from metabolism during oocyte maturation, it seems likely that the consumption of stored resources during thawing and recovery might be insufficient to sustain spindle integrity for prolonged periods of time unless some other resource were activated. This has been suggested by Jones *et al.* (2004), who showed a loss in mitochondrial integrity that was linked to defects in Ca^{2+} metabolism after cryopreservation. The energy requirements for spindle recovery would impact not simply energy used for tubulin polymerization but also on that required to maintain the activity of MT motors such as dynein to keep the spindle poles focused and also energy requirements for operating at the kinetochores that assure appropriate attachment of the chromosomes at the equatorial plate.

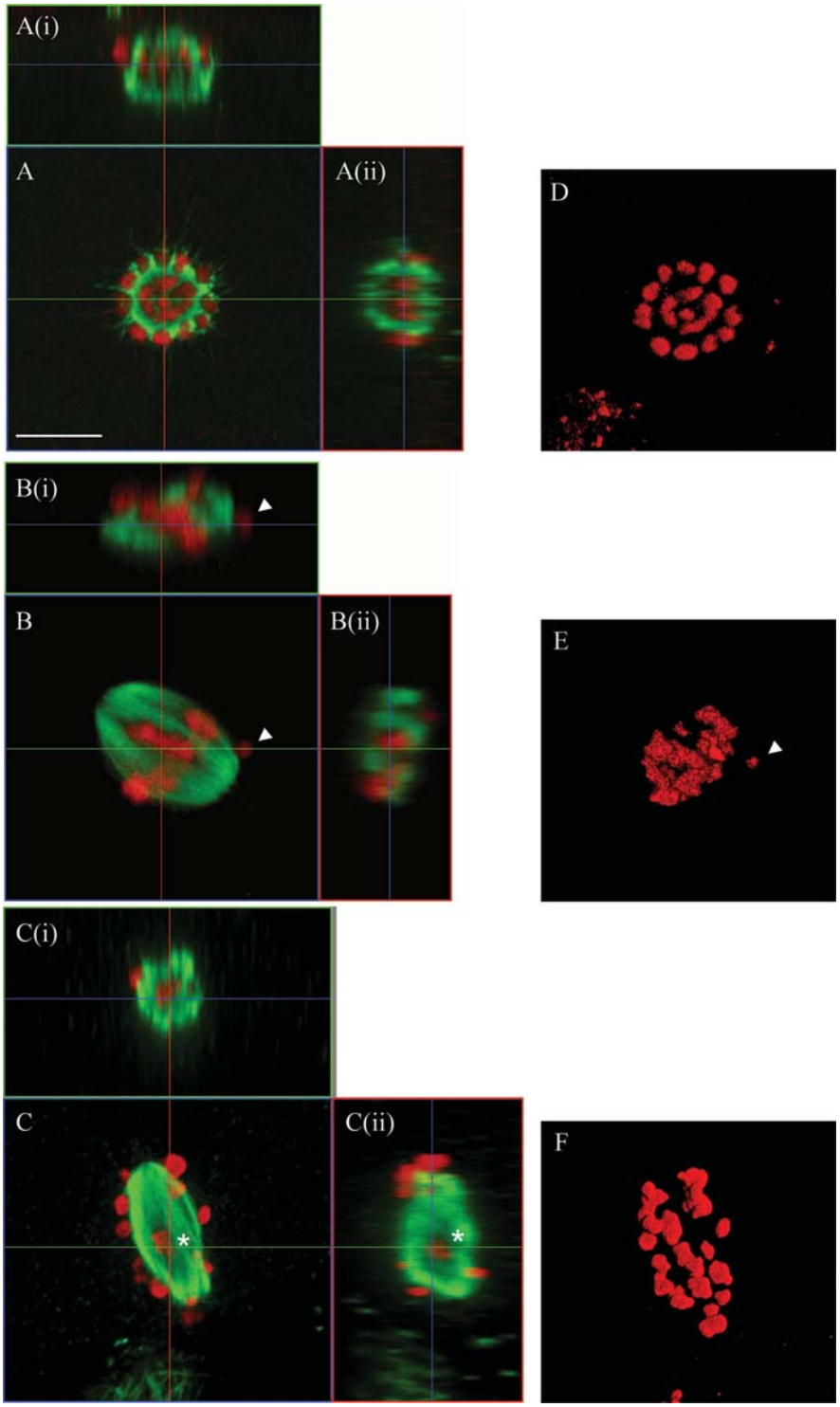


Figure 6 Representative confocal image showing 3-dimensional view of meiotic machinery.

Representative confocal images of bipolar spindles with aligned chromosomes (**A**), one displaced chromosome (**B**) and an elongated bipolar spindle with many displaced chromosomes (**C**). (A) Chromosomes are shown as being closely associated with MT bundles within the spindle structure itself in addition to peripheral bundles. (B) The majority of chromosomes aligned along the equatorial plate show a close interaction with MT bundles, however, the single displaced chromosome (B and Bi arrow heads) is loosely associated with MT surface. (C) Spindles with the majority of chromosomes displaced shows that most chromosomes interact with the spindle surface; note the presence of a single chromosome positioned at the equatorial plate and interacting with internal MT bundles (*). Three-dimensional representations of chromosome distribution from oocytes displayed in panel A, B and C are shown in panels D, E and F, respectively. Arrow head in E represents the displaced chromosome indicated by the arrow head in B, Bii. Total tubulin is shown in green and DNA in red. Scale bar represents 10 μm.

Other targets of putative cryopreservation-induced damage deserving of further study include the pool of tubulin heterodimers themselves as well as potential energy-generating components in biosynthetic pathways known to be present in the mature human oocyte (Kocabas *et al*., 2006). With respect to the tubulin pool, it is interesting to note that the overall mass of spindle MTs in human oocytes subjected to cryopreservation is considerably lower than that measured in unfrozen oocytes (Fig. 2). Whether this reflects a loss of protein functionality during the freezing process that limits the dynamic exchange of subunits during maintenance of the spindle is not known but does illustrate a useful problem on which proteomics may be brought to bear in future studies.

Although some combination of protein stability and metabolic factors are likely to contribute to the changes in meiotic spindle reformation and function observed after cryopreservation, the effect of maternal age on oocyte quality remains a dominant factor. Although these findings have implications for the timing of ICSI after thawing, this should not overshadow the importance of patient age in making such assessments. For example, we found a relationship between patient age and the spindle stability after thawing such that oocytes obtained from younger patients did not undergo spindle breakdown to the same extent as those from older patients. Thus, patient age should also be taken into account when making decisions as to the optimal time for performing ICSI in frozen thawed human oocytes.

More recently Polscope technology has become more prevalent in the IVF clinic and people have used this technology to dynamically trace the changes to the meiotic spindle following cooling or cryopreservation (reviewed in Keefe *et al*., 2003). Many of these studies suggest that human oocytes recover a functional spindle following 3 h of post-thaw culture, however, birefringence detected by the Polscope only dictates the presence or absence of MT bundles and is uninformative as to the spacing of chromosomes or the structural morphology of the spindle (Cottichio *et al*., 2009). In this regard we consider confocal analysis to be the most detailed and informative of technologies to conduct a study such as this, although the Polscope remains and important tool in specific processes in the clinic, such as ICSI. It should be pointed out however, that optimizing conditions for confocal analyses has revealed certain inconsistencies with our previous studies. Specifically, the incidence of apparently normal, bipolar spindles was higher in studies employing conventional fluorescence (Coticchio *et al*., 2006) versus confocal as used here. Moreover, refinement of data collection and analysis in the present study has allowed us to ascribe stricter criteria in classification of spindle polarity and chromosome alignment compared with the criteria adopted for classification in the De Santis *et al*. (2007) study from our group. Finally, we cannot exclude the possibility that variations between patient populations or oocyte quality could also have contributed to these apparent discrepancies. Nevertheless, these studies add to our understanding of the dynamics of human oocyte meiotic spindles after cryopreservation and serve as a point of departure for future studies using the criteria and approaches deployed here.

In conclusion, we have documented several important features of the human meiotic spindle as a result of carefully analysing its recovery and breakdown in slow-frozen and thawed oocytes using confocal microscopy and image analysis. These findings provide new and provocative insights into the unique character of the meiotic spindle that may help explain some of the functional deficits that have been associated with the origins of meiotic aneuploidy in the human. Moreover, there is a clear message of clinical importance here. The traditional use of 3 h recovery times following thawing of human oocytes needs to be re-examined in light of our findings and it is suggested that clinics consider earlier times after thawing for insemination of cryopreserved human oocytes in a patient age dependent context. Clearly, other cryopreservation protocols could expose oocytes to rather different conditions and should be investigated in a similar fashion on an individual basis.

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