Article

Vitrification may increase the rate of chromosome misalignment in the metaphase II spindle of human mature oocytes

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Abstract

The metaphase II (MII) spindle of the human oocyte may be damaged by cryopreservation. High performance confocal microscopy was used to assess meiotic spindle and chromosome organization in oocytes after vitrification by the cryoleaf system. Three hours after retrieval, donor mature oocytes were fixed or vitrified. Vitrification was performed by equilibration in 7.5% ethylene glycol (EG) and 7.5% dimethylsulphoxide (DMSO), transfer to 15% EG, 15% DMSO and 0.5 mol/l sucrose, and loading onto cryoleaf strips. Tubulin staining was found in all survived vitrified–warmed oocytes, the majority (62.8%) of which displayed a bipolar spindle. A normal bipolar spindle configuration and equatorial chromosome alignment was observed only in a part of vitrified–warmed oocytes (32.6%). This frequency was significantly lower in comparison to fresh oocytes (59.1%). In another fraction of vitrified–warmed oocytes (30.2%), spindle bipolarity was associated to one or more non-aligned scattered chromosomes that often appeared tenuously associated with the lateral microtubules of the spindle. Furthermore, in cryopreserved oocytes with a bipolar spindle, a significantly increased pole-to-pole distance (14.9 ± 2.3 µm) was found in comparison to the fresh control (12.4 ± 2.6 µm) ($P = 0.001$). Therefore, under the conditions tested, vitrified–warmed oocytes maintain a MII spindle with a bipolar organization. However, chromosome alignment appears to be partly compromised.

Keywords: *chromosomes, cryopreservation, oocytes, spindle, vitrification*

Several studies have suggested that the metaphase II (MII) spindle of the human oocyte may be exposed to damage caused by cooling (Pickering *et al.*, 1990), osmotic stress (Mullen *et al.*, 2004) and other physical or chemical conditions (Stachecki *et al.*, 1998) occurring during the return journey to and from physiological temperatures to cryogenic storage in liquid nitrogen (–196°C). This has generated the credence that oocytes cannot be safely cryopreserved, in consideration of an increased risk of errors in chromatid segregation during meiosis II, fertilization failure and other developmental anomalies. In reality, the question as to whether the oocyte cytoskeleton, and in particular the microtubular apparatus, is subjected to irreversible damage after cryostorage is rather complex and cannot be answered in simple terms (Bromfield *et al.*, 2009). Many factors, such as specific conditions imposed by individual cryopreservation protocols or elements that may influence oocyte quality (e.g. female age, culture conditions, manipulation *in vitro*) can act independently or through complex interactions, influencing the response of the cytoskeleton to cryopreservation in a fashion that is not always understood or even recognized. All the diverse cryopreservation approaches involve a potential risk of cell damage. Studies on the MII spindle of the human oocyte after cryopreservation have predominantly focused on the possible effects of controlled-rate slow-cooling protocols. Although the conclusions of these investigations have been not always consistent, at least some cryopreservation conditions do

not appear to affect the oocyte cytoskeleton and in particular the MII spindle (Gook *et al.*, 1993; Boiso *et al.*, 2002; Stachecki *et al.*, 2004; Bianchi *et al.*, 2005; Coticchio *et al.*, 2006; De Santis *et al.*, 2007). Evidence on oocytes cryopreserved by vitrification, a promising and rapidly expanding cryopreservation alternative, has been mainly generated in animal models (Chen *et al.*, 2001a; Cai *et al.*, 2005; Gomes *et al.*, 2008; Huang *et al.*, 2008). Data on human oocytes are still limited (Larman *et al.*, 2007; Cobo *et al.*, 2008a). On this basis, the present study used confocal microscopy to assess meiotic spindle organization, chromosome alignment and novel morphological parameters in oocytes after vitrification by the cryoleaf system.

Materials and methods

Oocyte collection and patient approval

This study was approved by the institutional review board of the participating clinic. Supernumerary oocytes were obtained from consenting couples undergoing assisted reproduction treatment for male or unexplained infertility. Ovarian stimulation was induced with long protocols using gonadotrophin-releasing hormone agonist and recombinant FSH, according to standard clinical procedures (Borini *et al.*, 2007). After retrieval and before cryopreservation, oocytes were cultured in Cook IVF Medium (Brisbane, Australia) for 3 h. After complete removal of cumulus cells, only oocytes showing normal morphology and an extruded first polar body (thus presumably at the MII stage) were selected and immediately fixed or vitrified. Oocytes were randomly assigned to the vitrified or control groups.

Oocyte vitrification, storage, warming and culture

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA). Vitrification and warming solutions were prepared in TCM199 (Sage IVF, Trumbull, CT, USA). Oocytes were equilibrated for 15 min at room temperature (RT) in equilibration solution containing 7.5% ethylene glycol (EG) and 7.5% dimethylsulphoxide (DMSO). Afterwards, they were placed for 1 min in vitrification solution, consisting of 15% EG, 15% DMSO and 0.5 mol/l sucrose, loaded onto cryoleaf strips (Medicult, Jyllinge, Denmark), and immediately submerged into liquid nitrogen until warming. Recovery from storage was conducted by direct transfer from liquid nitrogen to thawing solution (1.0 mol/l sucrose), at 37°C. After 1 min, oocytes were placed for 3 min at RT in dilution solution, including 0.5 mol/l sucrose, washed (5 min) twice in TCM199 and cultured for 1 h in glucose-free cleavage medium (Cook IVF or Sage IVF).

Oocyte fixation and immuno-staining

Oocytes were fixed fresh or after post-warming culture (Combelles *et al.*, 2002). To this end, oocytes were subsequently fixed in microtubule-stabilising buffer (100 mmol/l PIPES, 5 mmol/l $MgCl_2$, 2.5 mmol/l EGTA, 2% formaldehyde, 0.1% Triton X-100, 1 mmol/l taxol, 10 U/ml aprotinin and 50% deuterium oxide) for 30 min at 37°C and stored in wash solution (0.2% sodium azide, 2% normal goat serum, 1% bovine serum albumin, 0.1% glycine and 0.1% Triton X-100 in phosphate-buffered saline) until ready for further processing (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 at 4°C (all 1:100). Antibody detection was performed using a goat anti-mouse-alexa 488 (1:800, Molecular Probes, Invitrogen, USA) secondary antibody and 1 µg/ml of Hoechst 33258 (Molecular Probes) for DNA staining 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 including 50% glycerol/phosphate-buffered saline and 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 63x 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 three-dimensional projections for assignment of specific spindle and chromosomal properties (shape, length and polar constriction).

Oocytes were categorized as previously reported (De Santis *et al.*, 2007). Briefly, oocytes were categorised as having either a bipolar spindle with all chromosomes aligned (**Figure 1A**), a bipolar spindle with chromosomes displaced from the equatorial plate (**Figure 1B**), multipolar/ monopolar spindles in which chromosomes were aggregated (**Figure 1C**) or scattered throughout the microtubule structure (**Figure 1D**).

Statistical analysis

A chi-squared test was applied when comparing percentages. A Pearson correlation test was performed to determine the correlation between chromosomal displacement and spindle length. Spindle length was analysed using a one-way analysis of variance followed by a Student's *t*-test. A *P*-value <0.05 was considered statistically significant.

Results

The study was conducted including only oocytes showing an extruded first polar body (thus presumably at the MII stage) and normal morphology, i.e. spherical shape, absence of vacuoles and inclusions, and regular perivitelline space and zona pellucida. Overall, 65 oocytes from 27 patients were fixed and stained for total tubulin and chromatin (**Table 1**). There was no significant difference in patient ages between the two groups. One oocyte from each group was excluded from analysis as they were believed to have undergone parthenogenetic activation as shown by the restoration of cytoplasmic microtubules, chromatin decondensation and polar bodies, while one fresh oocyte apparently at the telophase stage was also not considered suitable.

Tubulin staining was found in all vitrified–warmed survived oocytes, the majority of which (27/43, 62.8%) displayed a bipolar spindle with focused poles (**Table 2**). However, a normal bipolar spindle configuration and equatorial chromosome alignment

(**Figure 1A**) was observed only in 32.6% of all vitrified–warmed oocytes (14/43). This frequency was significantly lower in comparison to the fresh group (59.1%). In another fraction of vitrified–warmed oocytes (13/43, 30.2%), spindle bipolarity was associated to one or more non-aligned, scattered chromosomes (**Figure 1B**). In such a case, chromosomes often appeared to jut out from the spindle outline. In 13/43 (30.2%) of vitrified–warmed oocytes, chromosome alignment was not accompanied by spindle bipolarity (**Figure 1C**), while in a small minority (3/43, 7.0%) both microtubule fibres and chromosomes were disarranged (**Figure 1D**).

Morphometric analysis also revealed a difference between treated and control groups. In fact, in cryopreserved oocytes with a bipolar spindle (**Figure 1A** and **B**), a statistically significant increase in pole-to-pole distance was found in comparison to the corresponding classes of fresh oocytes (14.9 ± 2.3 µm and 12.4 ± 2.6 µm, respectively; $P = 0.001$).

The study next examined the relationship between spindle length and chromosome position. Bipolar MII spindles from fresh or cryopreserved oocytes exhibited a wide range of chromosome displacement patterns, from equatorial plate alignment to

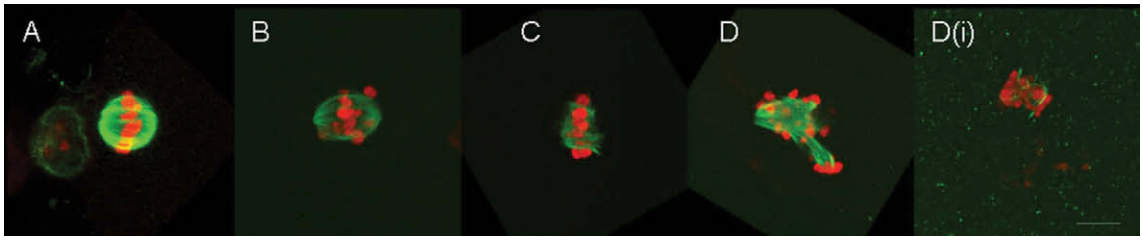

Figure 1. Representative confocal images of metaphase II spindles in vitrified and unfrozen oocytes. Meiotic spindles were classified as: (**A**) bipolar spindle with chromosomes aligned along the equatorial plate; (**B**) bipolar spindle with non-aligned, scattered chromosomes; (**C**) disarranged spindle 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 microtubule fibres persist and the chromosomes are aggregated into a mass. Note the lack of microtubule pole focusing and kinetochore bundling in **D**. Green = total tubulin; red = DNA.

Table 1. Number of donors, oocytes and age of donors.

Treatment	*No. of donors*	*No. of oocytes at MII*	*No. of MII oocytes from each patient*	*No. of oocytes at telophase (% of total)*	*No. of activated oocytes (% of total)*	*Age (years)*[a]
Unfrozen	6	22	1, 2, 3, 3, 4, 9	1 (4.5)	1 (4.5)	37.5 ± 2.0 (30–43)
Vitrified	21	43	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 2, 2, 3, 3, 4, 4, 11	0 (0.0)	1 (2.3)	38.5 ± 0.8 (29–43)

Only oocytes analysed for total tubulin are included. MII = metaphase II.

[a]Values are mean ± SEM (range).

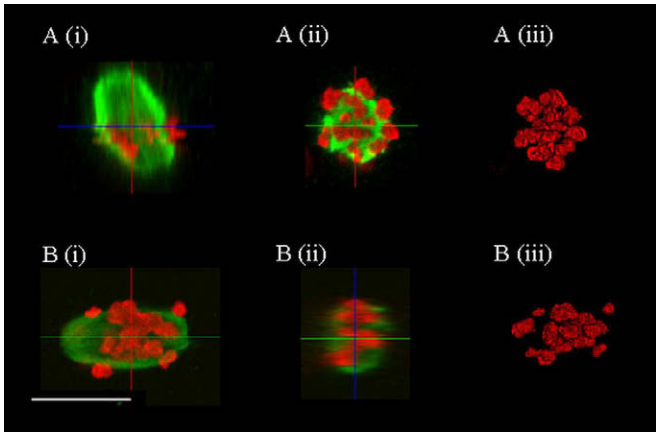

Figure 2. Representative confocal images of (**A**) bipolar spindle with aligned chromosomes and (**B**) an elongated bipolar spindle with many displaced chromosomes. In **A(i)** and **A(ii)**, chromosomes are shown as being closely associated with microtubule bundles within the spindle structure itself in addition to peripheral bundles. In **B(i)** and **B(ii)**, spindles with the majority of chromosomes displaced shows that most chromosomes interact with the spindle surface. Three-dimensional representations of chromosome distribution are shown in panels **A(iii)** and **B(iii)**. Green = total tubulin; red = DNA. Bar = 10 µm.

Table 2. Percentages of the different spindle classes in vitrified and unfrozen oocytes.

	A	*B*	*C*	*D*	*Di*
Unfrozen	13/22 (59.1)	9/22 (40.9)	0/22 (0.0)	0/22 (0.0)	0/22 (0.0)
Vitrified	14/43 (32.6)	13/43 (30.2)	13/43 (30.2)	3/43 (7.0)	0/43 (0.0)

Values are number/total (percentage). Spindle classes as defined in **Figure 1**.

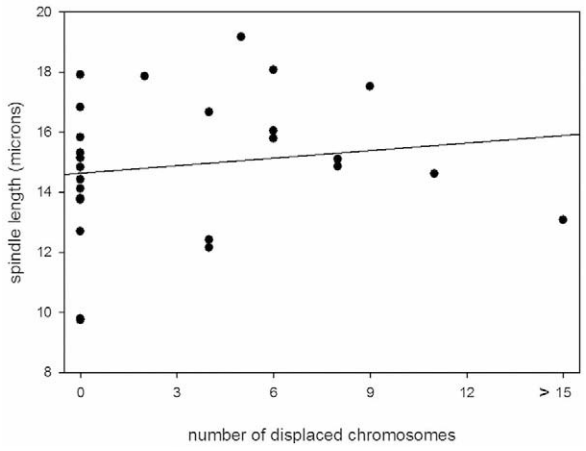

Figure 3. Scatter plot showing the number of dispersed chromosomes in relation to the pole-to-pole length of individual bipolar spindles. Each dot represents one spindle including those with no displaced chromosomes (n = 27). The Pearson correlation test gave an r-value of 0.15, which was not statistically significant.

widespread scattering (**Figure 2**). In cases of complete chromosome alignment, two concentric rings of chromosomes were evident occupying indentations in the compact spindle. Not uncommonly, in other cases chromosomes were found dispersed and tenuously bound. Based on their number and relative size, it appeared that displaced chromosomes represented sister chromatid pairs that had not disconnected, implying that the trend towards elongation was not due to precocious activation of the cell cycle or anaphase onset.

Because the number of displaced chromosomes varied between samples, spindle lengths were examined as a function of how many chromosomes failed to remain in the metaphase plate. No statistically significant association was found between increased spindle length and number of displaced chromosomes (**Figure 3**). Interestingly, however, displacement of more than four chromosomes was always associated with spindle length higher than the mean observed in the fresh control (12.4 ± 2.6 µm).

Conclusions

Because of its inherent dynamic properties (Howard and Hyman, 2003) and sensitivity to a variety of intrinsic and extrinsic factors, such as age (Battaglia *et al.*, 1996) and low temperatures (Pickering and Johnson, 1987), the MII spindle of the human oocyte has been suspected to be a potential target of adverse cryopreservation conditions, with possible implications for the health of children developed from cryopreserved oocytes. This issue has attracted considerable interest, leading to several studies conducted on human and animal model oocytes. Historically, epifluorescence microscopy has been instrumental to ascertain whether the canonical bipolar spindle organization and equatorial chromosome alignment is preserved after cryopreservation (Gook *et al.*, 1993). More recently, technical and methodological advances in confocal microscopy have integrated previously unrecognized morphometric parameters of normalcy with the traditional assessments of spindle bipolarity and chromosome alignment (Bromfield *et al.*, 2009). These observations will expand the understanding of the organization and function of MII spindle, irrespective of the concerns raised by the possible consequences of cryopreservation. Various studies indicate that preservation of spindle integrity is compatible with some controlled-rate slow-cooling methods (Gook *et al.*, 1993; Stachecki *et al.*, 2004; Coticchio *et al.*, 2006; Larman *et al.*, 2007; Cobo *et al.*, 2008a). Overall, however, it appears difficult to draw general conclusions because even relatively minor protocol modifications, such as diverse dehydration conditions (Mullen *et al.*, 2004; Coticchio *et al.*, 2006) or type of storage device (Chen *et al.*, 2001b), can have different and unpredictable downstream effects on the process of cryopreservation and therefore on microtubule organization. This imposes the need for testing individually each cryopreservation protocol, especially those newly developed.

In recent years, the vitrification approach has emerged as a potential solution to the problem of oocyte cryopreservation, achieving in the most successful cases very high recovery rates and percentages of fertilization and pre- and post-implantation development that parallel those of fresh oocytes (Kuwayama *et al.*, 2005; Lucena *et al.*, 2006; Antinori *et al.*, 2007; Cobo *et al.*, 2008b; Konc *et al.*, 2008; Kusakabe *et al.*, 2008). However, it remains to be established whether different vitrification conditions are equivalent in relation to their ability to preserve the cellular constitution of the oocyte and ensure a high clinical outcome. Following vitrification, the oocyte MII spindle has been investigated principally in rodents. In the mouse, it appears that the type of storage device can influence both

survival and spindle integrity, closed pulled straws having been shown to perform better than open pulled straws, conventional straws and grids (Chen *et al.*, 2001b). A recent study has confirmed that vitrification by closed pulled straws leaves spindle and chromosome organization unaffected in mouse oocytes. Other factors, such as a high cooling rate achievable with special vitrification machinery (Vit Master), have been reported to be critical to preserve spindle bipolarity and normal chromosome alignment in rabbit oocytes (Cai *et al.*, 2005). These experiences have represented important steps towards the validation of vitrification as a tool for human IVF treatment, but have not provided a final answer to the question of possible spindle damage after vitrification in the human, in light of the different capability by which rodent and human microtubules can reorganize after cooling-induced depolymerization (Pickering and Johnson, 1987; Pickering *et al.*, 1990).

Oocyte cryopreservation has also been investigated by polarized light microscopy that offers the opportunity to visualize the meiotic spindle non-invasively and in a dynamic fashion, thereby opening a unique opportunity for studying valuable human material. Orderly structures of spindle microtubules generate the phenomenon of birefringence that creates a difference in contrast between the spindle and the rest of the cell. This may be detected by imaging methods (e.g. the PolScope) that amplify birefringence signals and make quantifiable the degree of microtubule orientation (Keefe *et al.*, 2003). Using the PolScope, Larman *et al.* (2007) reported that in oocytes stored by cryoloop vitrification, but not controlled-rate slow-cooling, spindle birefringence was detectable immediately after warming. It should be noted, however, that this study tested a notoriously inefficient and obsolete controlled-rate slow-cooling protocol (Borini *et al.*, 2004). Recently, Ciotti *et al.* (2008) have extended the findings of Larman *et al.* (2007), concluding that the MII spindle undergoes depolymerization as an effect of cryopreservation and that spindle birefringence is recovered more rapidly after vitrification at 37°C in comparison to controlled-rate slow-cooling conducted at room temperature. The findings of these authors are of significant importance for the study of spindle dynamics after cryopreservation. Nevertheless, it should be noted that the PolScope has limited predictive value of the degree of spindle fibre order and chromosome position (Coticchio *et al.*, 2009). This suggests that the PolScope may be a rather inefficient method for assessing the oocyte MII spindle.

Under the vitrification conditions tested in this study, it was found that in the majority of cryopreserved oocytes the MII spindle maintains a bipolar organization. However, chromosome alignment appears to be compromised in approximately 50% of bipolar spindles. These findings are in contrast with those recently reported by Cobo *et al.* (2008a), who observed that the proportion of oocytes showing a bipolar spindle and equatorial chromosome alignment was unaltered after cryotip vitrification. Cao *et al.* (2008) have also reported that the incidence of appropriate spindle integrity and chromosome alignment is comparable between fresh and vitrified human oocytes. There are no obvious explanations for these discrepancies, although some hypotheses may be formulated. For instance, it was recently observed that oocytes from older patients appear to possess a reduced ability to reform and maintain over time a normal spindle after cryopreservation (Bromfield *et al.*, 2009). The data of Cobo *et al.* (2008a) were generated from oocytes of young women (mean age 25.1 years) and therefore are perhaps not directly comparable with this study, considering the much higher mean age of this study's patients (38.5 years). The mean patient age was not described in the work of Cao *et al.* (2008). Another factor that might explain the diversity in the results of these studies consists in the different time points at which vitrified and thawed oocytes were fixed for confocal examination: 2–3 h in the analyses of Cobo *et al.* (2008a) and Cao *et al.* (2008) and 1 h in this study. This has relevance to the fact that it was recently reported that the meiotic spindle can undergo complex dynamics of microtubule rearrangements during the first few hours following thawing (Bromfield *et al.*, 2009). Therefore, one single observation after thawing–warming might be insufficient to describe accurately the condition of the oocyte MII spindle after cryopreservation. The importance of the time factor is confirmed the work of Gomes *et al.* (2008) who observed that in mouse oocytes vitrified with closed pulled straws as a storage device the meiotic spindle may be lost immediately after warming but reappears apparently unaltered immediately after culture at 37°C for 2 h. Nevertheless, it should be noted that this animal model is perhaps not ideal for the meiotic spindle of human oocytes considering that the ability of microtubules to reorganize following depolymerization caused by lower temperatures is rather more pronounced in the mouse (Pickering and Johnson, 1987; Pickering *et al.*, 1990).

From this analysis, it also appears that a certain proportion of vitrified oocytes shows rather atypical features, i.e. a tendency towards spindle elongation and protrusion of chromosomes outside the spindle. The increase in pole-to-pole distance was accompanied by an apparent reduction in width. By analogy to recent observations in oocytes cryopreserved with a controlled-rate slow-cooling protocol, this is suggestive of a decreased total mass of spindle tubulin (Bromfield *et al.*, 2009). The significance of these manifestations is still uncertain, although is it reasonable to suspect that they may represent an indication for an increased risk of meiotic errors. Further studies are warranted in this area to expand understanding of the nature of possible influences on the integrity of the meiotic spindle generated by the process of vitrification. In fact, the concept that mere spindle bipolarity and chromosome alignment are not sufficient to define spindle normality is starting to emerge (Sanfins *et al.*, 2003; Bromfield *et al.*, 2009). Studies are in progress to ascertain whether the anomalies observed in oocytes vitrified by cryoleaf are recoverable during post-warm culture, and therefore only transiently expressed, or persist after warming. Other analyses will need to be performed especially on cryotop vitrification methods, in the consideration that clinical trials currently in progress rely in most cases on the use of this storage device. This may be not without consequences, because the design of the containment device influences the volume in which oocytes are vitrified, with possible effects on the rate of cooling and warming and, in final analysis, on the efficiency by which the biological material is preserved.

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