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ARTICLE

Slow-freezing-induced changes of birefringent structures in human oocytes are related to responsiveness to ovulation induction

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Emanuela Molinari obtained her degree in Biotechnology in 2007 at the University of Turin, Italy. She carried out her training as clinical embryologist at the Italo-Swedish Assisted Reproduction Clinic LIVET in Turin, and graduated via a thesis on human oocyte evaluation with polarized light. In 2008 she started her PhD in Clinical Sciences at the IVF Laboratory of the St Anna Hospital, University of Turin Medical School. The research areas in which she is currently involved are gamete and embryo cryopreservation, gamete selection and preimplantation diagnosis.

Abstract The slow-freezing method is widely used to freeze human oocytes, both for fertility preservation and in routine IVF programmes. Slow freezing damages some of the cell's structures, including the meiotic spindle (MS) and the zona pellucida (ZP). Polarized light microscopy was used to study the variations induced by slow freezing on the MS and the ZP of human oocytes and to analyse the relationship between slow-freezing effects on the gamete and some clinical characteristics, such as age, body mass index and ovarian responsiveness to ovulation induction (expressed as total follicle-stimulating hormone dose/retrieved oocyte). Both the MS and the ZP (particularly its inner layer) underwent significant changes during slow-freezing procedure. The MS became thinner and structurally less organized (lower retardance) ($P < 0.001$ and $P < 0.05$, respectively), whereas the ZP became thicker and its inner layer lost structural organization (both $P < 0.05$). These morphological changes were unrelated to the patient's age or body mass index, but ZP variations in thickness and retardance were significantly related to ovarian responsiveness ($P = 0.033$ and $P = 0.026$, respectively), suggesting that patients with a higher response to gonadotrophins produce oocytes better able to preserve their characteristics after freezing–thawing.

KEYWORDS: meiotic spindle, oocyte cryopreservation, oocyte freezing, polarized light microscopy, zona pellucida

Introduction

Oocyte cryopreservation is considered a useful tool to preserve female fertility before antiblastic therapy (Fabbri, 2006; Manipalviratn and Decherney, 2008; Porcu et al., 2004) and, in countries where legal rules forbid or strongly limit embryo freezing, it is also widely used in routine IVF. The slow-freezing–rapid-thawing method proposed by Fabbri et al. (1998) is still one of the most popular techniques to cryopreserve human oocytes, although oocytes

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cryopreserved by this method have a lower potential in terms of pregnancy chance in comparison to fresh eggs (Borini et al., 2006, 2007; De Santis et al., 2007a,b; Oktay et al., 2006; Parmegiani et al., 2009). This loss of effectiveness is mainly due to the damage induced by the slow-freezing procedure on some of the key structures of the cell, including the meiotic spindle (MS) and the zona pellucida (ZP) (Wininger and Kort (2002)). These structures are of utmost importance in the reproductive process, the first regulating chromosomal alignment and distribution during meiosis, the latter regulating sperm—egg interaction as well as blastocyst hatching.

Polarized light microscopy (PLM) allows the non-invasive detection of both the MS and the ZP, that appear birefringent when illuminated with polarized light. Recently some authors have suggested that the birefringent properties of these structures can be used to predict oocyte developmental potential after thawing (Rama Raju et al., 2007; Shen et al., 2005, 2006).

This study used PLM to study the variations induced by the slow-freezing procedure on the MS and the ZP of mature (metaphase II; MII) human oocytes and analysed the relationship between the effects of slow freezing on the gamete and some clinical characteristics of the patient, such as age, body mass index (BMI) and ovarian responsiveness to hormonal stimulation.

Materials and methods

Oocyte collection

The study was authorized by the local ethical committee and all patients gave their written consent to use some of their oocytes for research purposes.

A total number of 152 MII oocytes were collected from 48 patients undergoing IVF in the study in the year 2008, when the Italian law on assisted reproduction allowed use of only three mature oocytes for fertilization. The oocytes used in this cohort observational study were spare oocytes donated by the patients and were not submitted to IVF or ICSI to obtain a pregnancy. They were chosen randomly among the cohort of MII oocytes with clear cytoplasm, no granulation or inclusions that were available in each patient's retrieval.

Ovulation induction was induced using a standard long protocol with gonadotrophin-releasing hormone agonist (Buserelin) plus gonadotrophins (recombinant follicle-stimulating hormone, rFSH; Merck-Serono, Geneva, Switzerland) at appropriate doses (100—450 IU), that were estimated according to the woman's age, the antral follicle count and the basal (day 3) FSH concentration. Ovarian response to stimulation was monitored by transvaginal ultrasound plus serum oestradiol measurement every third day from stimulation day 7. Ovulation was triggered by administration of 10,000 IU human chorionic gonadotrophin (Ibsa, Lugano, Switzerland) i.m. when the leading follicle reached 18 mm, with appropriate serum oestradiol concentrations. Transvaginal ultrasound-guided oocyte aspiration was performed approximately 36 h after human chorionic gonadotrophin injection under local anaesthesia (paracervical block). After retrieval, the eggs were incubated in IVF culture medium (IVF; MediCult, Denmark) for at least 3 h. Then the corona—cumulus—complex was removed with hyaluronidase (SynVitro Hydase 80 IU/ml; MediCult) and repeatedly aspirating oocytes with micropipettes (Cook, Australia).

The patient's responsiveness to rFSH was assessed by dividing the total administered FSH dose by the number of eggs retrieved. Since the ovulation induction protocol was similar for all patients and retrieval was always performed by the same four operators without appreciable inter-operator technical differences, the number of FSH units per retrieved oocyte gives an accurate idea of the ovarian sensitiveness to hormonal stimulation.

Slow-freezing procedure

Three hours after cumulus removal, oocytes were cryopreserved using the slow-freezing protocol described by Fabbri et al. (1998). All media used for cryopreservation were previously warmed at room temperature (22.5—23°C) for at least 1 h. Oocytes were washed in phosphate-buffered saline supplemented with 30% human serum albumin (HSA), placed for 10 min in the first freezing solution (1.5 mol/l 1,2 propanediol; 1,2-PROH), then moved to the second solution for 1 min (1.5 mol/l 1,2-PROH + 0.3 mol/l sucrose). After this step, oocytes were loaded individually in plastic straws (Paillettes Crystal 133 mm; Cryo Bio System, France), transferred into an automated vertical freezer (Kryo 10 Series III, Planer Kryo 10/1.7 GB; Planer, UK) and frozen according to the following conditions: start chamber temperature 20°C; slow cooling to −8°C at a speed rate of −2°C/min; ice seeding performed manually at −8°C; cooling to −30°C at a speed rate of −0.3°C/min; cooling to −150°C at a speed rate of −50°C/min; and loading the straws in liquid nitrogen.

Thawing procedure

After a maximal time of 1 month of cryopreservation, the straws were exposed for 30 s to room temperature and then placed in pre-warmed water (30°C) for 40 s. The cryoprotectant was removed by stepwise solutions containing decreasing concentrations of 1,2-PROH. The oocytes were placed for 5 min in the first solution (1 mol/l 1,2-PROH + 0.3 mol/l sucrose + 30% HSA) and then placed for further 5 min in the second solution (0.5 mol/l 1,2-PROH + 0.3 mol/l sucrose + 30% HSA). Finally, they were placed in a 0.3 mol/l sucrose + 30% HSA solution for 10 min, and at 37°C for further 10 min. Once the thawing procedure was completed, oocyte viability was assessed by observing some morphological characteristics as previously published (De Santis et al., 2007a,b).

PLM analysis

PLM measurement of MS and ZP birefringent characteristics was performed by LC Polscope optics combined with a computer-assisted image analyser (Spindle View; CRI, Woburn, MA, USA). Before freezing and after thawing, each oocyte was placed in a glass-bottomed culture dish (WillCo Wells, Amsterdam, The Netherlands) in a 10 μl drop of buffered medium (Flushing Medium, MediCult; Denmark) covered by 5 ml of mineral oil (Liquid Paraffin, MediCult). The dishes were maintained at 37°C during PLM examination and the

oocytes were manipulated using a holding pipette (Swemed, Sweden) in order to rotate them until the MS was at 12 o'clock at 400× magnification. Thawed oocytes were analysed after 3 h of incubation at 37°C in order to allow complete repolymerization of the MS as previously suggested (Bianchi et al., 2005). MS thickness and light retardance were measured performing a line scan across the MS long axis by gradual image magnification and by calculating the mean value of the line (Coticchio et al., 2009) (**Figure. 1**). A line scan across the entire ZP was performed to measure its thickness and retardance. To provide better accuracy, the ZP was zoomed in the line scan area, and considering the irregularity of the structure, four different points (at 3, 6, 9 and 12 o'clock) were measured (Shen et al., 2005) and the average value was calculated. When measuring ZP retardance, three peaks were obtained, each representing the highest value of the inner, middle and outer ZP layers (**Figure 2**); both the retardance of each single layer and the mean value of the three ZP layers taken together were measured.

Statistical analysis

The two tailed Student's *t*-test for paired data was used to compare the values of thickness and retardance of both ZP and MS before and after freezing. The linear regression analysis was used for evaluating the relationship between

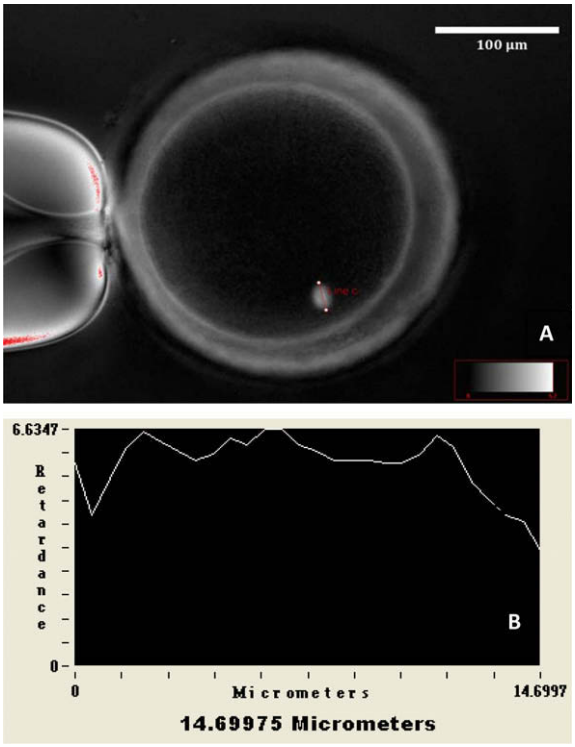

Figure 1 Polarized light microscopy measurement of the thickness and the retardance of the meiotic spindle (MS) of human oocytes. (**A**) A line scan across the MS long axis is performed and (**B**) MS birefringent parameters are objectively measured. The retardance of the spindle is calculated as the mean value for the line and expressed as nanometers (nm). Bar = 100 µm (**A**).

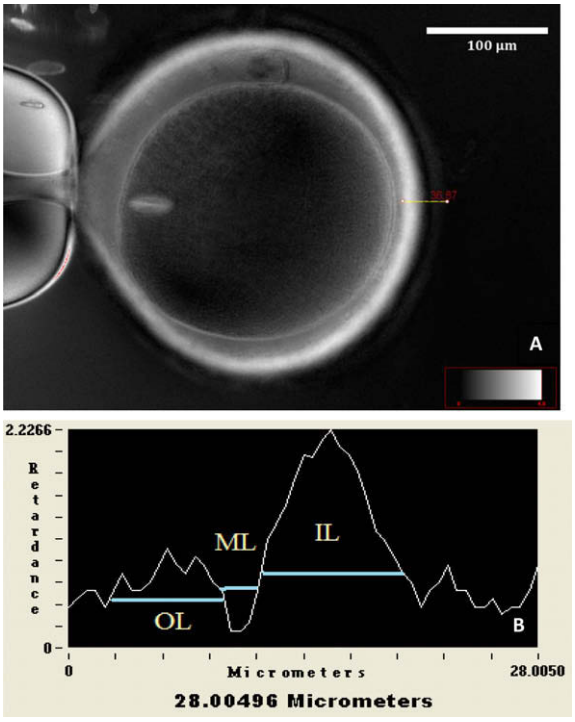

Figure 2 Polarized light microscopy measurement of the thickness and the retardance of the three layers of the zona pellucida (ZP) of human oocytes. (**A**) A line scan across the three inner layers is performed and (**B**) MS birefringent parameters are objectively measured. Each of the three peaks in (**B**) represents a single layer of the ZP starting from the outer (OL) through the middle layer (ML) to the inner layer (IL). The retardance of the entire ZP is calculated as the mean of the three values and expressed as nanometers (nm) (**B**).

PLM parameters and the patients' clinical characteristics. Since all patients donated more than one oocyte (from two to six) for this study, in order to have the same number of observations ($n = 48$) on both axis of the linear regression, the data of the oocytes retrieved from each patient were pooled and their mean was used for regression analysis. A value of $P < 0.05$ was considered statistically significant.

Results

Forty-eight patients (age 26—43 years, BMI 15.6—29.4 kg/m^2) underwent ovulation induction with rFSH obtaining a total number of 376 mature oocytes (range 5—16 per patient); the median rFSH dose used for ovulation induction was 2478 IU (range 1725—4050) and the FSH dose/retrieved oocyte ranged from 215 to 675 IU. None of the patients submitted to ovarian stimulation needed a significant dose adjustment during stimulation; this suggests that the administered FSH dose, chosen on the basis of age, antral follicle count and basal FSH concentration, was appropriate to retrieve enough oocytes (from five to 16) to allow fertilization and embryo transfer on one side, egg donation for research on the other side. Further, none of the patients appeared to be overstimulated or understimulated, as the number of retrieved oocytes and the peak oestradiol concentration (1540—3050 pg/ml) were in the normal range for human IVF.

A total of 152 oocytes were donated for research purposes and were used in the study. These oocytes were frozen by the slow-freezing procedure and the overall oocyte survival rate after freezing–thawing procedure was 86% ($n = 131$). At least one oocyte per patient survived after thawing (mean ± SD 4.5 ± 2.45).

After thawing, the spindle remained detectable in 84% ($n = 110$) of the survived oocytes; thus, calculations on PLM parameters were performed comparing data on these 110 oocytes observed before and after slow freezing.

Both MS thickness and MS retardance were significantly higher in fresh than in thawed oocytes (**Table 1**). The extent of spindle thinning after cryopreservation (change in MS thickness) was significantly related to ovarian responsiveness to FSH: patients who needed more FSH/oocyte (lower ovarian responsiveness) showed a more marked effect of freezing on MS thickness ($P = 0.033$); this was not observed for change in MS retardance, that was not significantly related to ovarian responsiveness. Both changes in MS thickness and retardance were not significantly related with the patients' age and with BMI.

The ZP thickness increased significantly after freezing ($P < 0.05$), whereas its retardance did not significantly change (**Table 1**). The changes in ZP thickness and retardance were not significantly related to the patient's age, BMI and ovarian responsiveness to gonadotrophin stimulation. However, considering only the inner layer of the ZP, a significant thickening after freezing–thawing and also a significant reduction of retardance (both $P < 0.05$; **Table 1**) were observed. The changes of the inner layer's thickness and retardance were significantly related to the ovarian responsiveness to FSH ($P = 0.033$ and 0.026, respectively), but not with the patients' age and BMI. Patients needing a higher FSH dose/oocyte showed a more marked post-freezing increase of the inner layer's thickness and a more marked decrease of its retardance.

Discussion

The birefringence of the MS and the ZP of human oocytes is due to optical properties of some molecules that constitute these structures. When polarized light hits ordered molecules, such as the glycoproteins forming the zona or the microtubule proteins of the spindle, part of the light is retarded passing through the structure and this generates what is called retardance. PLM measures both the distance between two definite points (i.e. spindle or zona thickness) and the retardance of the structure, which roughly reflects its structural order. In fact, the more ordered is the structure, the more light diverges and generates greater retardance: MS retardance has been reported to be linked to the density and order of its microtubules (Keefe et al., 2003). With the normal imaging techniques used in IVF laboratories, visualizing the meiotic spindle is impossible as the contrast is too low; staining methods allow optimal spindle visualization, but are not compatible with using the oocyte to get a pregnancy. An important value of PLM is that it allows MS visualization without affecting oocyte viability and allowing it to be used to obtain an embryo suitable for transfer *in utero*.

It has been shown that the slow-freezing–thawing procedure has detrimental effects on the MS: freezing causes MS depolymerization in practically all oocytes (Coticchio et al., 2006; Rienzi et al., 2004, 2005), and even if repolymerization occurs in most of them within a few hours after thawing (Bianchi et al., 2005; Cobo et al., 2008), it may not be sustained at later times, finally resulting in an altered MS, unable to correctly allow chromosomal alignment and distribution to daughter cells (Bromfield et al., 2009). It must be acknowledged that different cryopreservation techniques, such as vitrification, are known to cause less damage to the oocytes and a faster post-thaw spindle recovery (Ciotti et al., 2009; Ko et al., 2008; Larman et al., 2007); as a consequence, the results observed for slow freezing may not be observed in cases where vitrification is used.

This study observed a significant decrease in MS thickness and retardance after thawing, data that are consistent with those recently obtained in electron microscopy studies (Nottola et al., 2007, 2008). MS retardance measured by PLM has been claimed to reliably indicate oocyte quality (Shen et al., 2006), but a recent study questioned its value, as the comparison with the results of confocal microscopy showed that PLM-measured MS retardance is not precise in detecting the degree of spindle fibre order (Coticchio et al., 2009). Differently, MS thickness was recently associated with a favourable outcome in terms of oocyte fertilization and subsequent embryo progression to blastocyst (Rama Raju et al., 2007); according to this observation, it can be speculated that limiting MS thinning after slow freezing could reflect the ability of the oocyte to preserve its potential. Interestingly, the effects of freezing–thawing on the MS are significantly less marked when the ovary shows a higher level of responsiveness to hormonal stimulation, independent of the woman's age and BMI. It is widely

Table 1 Thickness and retardance of the meiotic spindle, the zona pellucida and its inner layer before and after the slow-freezing–thawing procedure in 110 oocytes.

Characteristic	*Before freezing*	*After thawing*	P-*value*
Meiotic spindle thickness (μm)	11.73 ± 1.93	9.99 ± 2.87	<0.001
Meiotic spindle retardance (nm)	2.23 ± 0.68	1.98 ± 0.63	<0.05
Zona pellucida thickness (μm)	27.73 ± 3.41	29.78 ± 4.36	<0.05
Zona pellucida retardance (nm)	1.52 ± 0.37	1.39 ± 0.63	NS
Inner-layer thickness (μm)	7.18 ± 0.77	8.00 ± 0.76	<0.05
Inner-layer retardance (nm)	2.85 ± 0.86	2.19 ± 0.70	<0.05

Values are mean ± SD. Significance is calculated using the two tailed Student *t*-test for paired data. NS, not statistically significant.

documented that a worse responsiveness to exogenous gonadotrophins in IVF is associated with the production of lower quality oocytes and more likely to generate aneuploid embryos (Gianaroli et al., 2000). It is shown herein that the MS structure of the oocytes retrieved from good FSH-responsive patients undergoes smaller changes than that of the oocytes of less responsive women. The MS of the oocytes retrieved from good responders seems to be more resistant to the depolymerization-inducing physical stress: this could contribute to the reported lower aneuploidy rate of the embryos in this subset of patients (Gianaroli et al., 2000).

The PLM study of the ZP showed an overall thickening after slow freezing. The increase of ZP thickness after freezing–thawing has been observed in early studies as a consequence of the premature extrusion of cortical granules (Vincent et al., 1990). The current study reports that ZP thickening is more pronounced in oocytes coming from patients having a lower responsiveness to gonadotrophins, whereas neither age nor BMI appear to be related to it. Once again, it can be speculated that a better preservation of ZP thickness after freezing in good responders could reflect a better oocyte quality and, maybe, a better chance for the derived embryo to hatch through a thinner zona and implant *in utero*.

The inner layer of the ZP appears to be the most important part of the zona and the only one that could be proposed as a marker of oocyte quality. According to previous reports, in fact, a thicker inner layer and a higher inner-layer retardance are associated with a better oocyte competence to generate good-quality embryos and lead to conception cycles (Rama Raju et al., 2007; Shen et al., 2005). The present study observed that, during slow freezing, the inner layer undergoes a significant thickening as well as a significant reduction of retardance, probably reflecting a higher degree of structural disorganization. If retardance is positively related to oocyte health and to the conception chance, this loss of retardance could just be a morphological sign of the worsening quality of the fresh oocyte when submitted to freezing.

These data suggest that the inner part is probably the most cooling-sensitive part of the ZP, since considering the ZP as a whole as well as the middle and outer layers singularly, retardance was not significantly affected by slow freezing. It was found that also the reduction of inner-layer retardance was significantly related to ovarian responsiveness (being more pronounced in oocytes coming from patients with poor response to FSH), but was unrelated to the patient's age and BMI.

In conclusion, this study on the effects of slow-freezing technique on human oocytes evaluated by PLM showed that both the MS and the ZP (particularly its inner layer) undergo significant changes during freezing–thawing. The MS becomes thinner and structurally less organized, the ZP becomes thicker and its inner layer loses structural organization. These morphological features are not related to the patient's age or BMI, but are significantly linked to the level of ovarian responsiveness to ovulation induction, suggesting once again that patients with a higher response to exogenous FSH produce better-quality oocytes, able to better preserve their potential after the freezing–thawing procedure.

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