Cryopreservation of metaphase II human oocytes effects mitochondrial membrane potential: implications for developmental competence

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BACKGROUND: Current outcome results with embryos derived from thawed MII human oocytes are significantly lower than with embryos cryopreserved at the pronuclear stage. Here, we investigated whether freezing–thawing was associated with changes in oocyte mitochondrial polarity (ΔΨm) that could influence competence by altering ATP levels or the ability of the cytoplasm to regulate intracellular Ca2+. METHODS: Fresh and thawed uninseminated and unfertilized MII oocytes were stained with the ΔΨm-specific probe JC-1 to detect clusters of high-polarized mitochondria (J-aggregate positive) and with the Ca2+ specific probe Fluo-4 to measure changes in intracellular levels of this cation. ATP content per oocyte was measured directly and cortical granules were visualized with a cortical granule-specific probe. RESULTS: A significant difference between fresh and thawed MII oocytes existed for pericortical J-aggregate fluorescence and for the ability of the cytoplasm to increase free Ca2+ in response to ionophore exposure. No significant difference in ATP contents was measured and cryopreservation was not associated with an apparent release of cortical granules. CONCLUSION: Irreversible loss of high ΔΨm in thawed oocytes may be associated with defects in Ca2+ signalling after insemination and could have downstream consequences for normal embryogenesis.

Key words: ATP/calcium/embryo competence/mitochondrial polarity/oocyte cryopreservation

Introduction

Although controversial (Winston, 2002), oocyte cryopreservation is largely considered the most realistic method currently available to preserve the fertility potential of women experiencing premature ovarian failure or facing certain sterility resulting from chemotherapy (Chen et al., 1986; Gook et al., 1994, 1995; Tucker et al., 1996, 1998a,b; Porcu, 2001; Boldt et al., 2003; Fosas et al., 2003). However, outcomes from several thousand attempts of ICSI with thawed metaphase II (MII) human oocytes demonstrate high frequencies of fertilization and early development (Gook et al., 1995; Tucker, 1996; Tucker et al., 1998a) but low implantation rates, with approximately one-third of implantations spontaneously aborting (Porcu, 2001) and term pregnancy rates reported in the single digits (Ludwig et al., 1999). Recent modifications to cryosolution composition and the duration of dehydration have been reported to increase survival rates for oocytes and improve outcomes for resulting embryos (Boldt et al., 2003; Fosas et al., 2003). However, while fertilization and cleavage rates after ICSI are typically >50% with these oocytes, ongoing pregnancy rates remain well below levels routinely obtained (>35%) with thawed pronuclear embryos where the same cryoprotectants and cooling protocol are used (Queenan et al., 1997; Damario et al., 2000).

While the underlying cause(s) of poor outcomes with thawed human oocytes are unknown, numerical chromosomal disorders (aneuploidy and mosaicism) resulting from cooling-induced meiotic spindle defects are generally assumed to be the primary factor adversely affecting embryo competence (Pickering et al., 1990; Almeida and Bolton, 1995; Wang et al., 2001). However, several studies report no significant increase in levels of chromosomal disruption after thawing of human oocyte (Gook et al., 1993, 1994; Van Blerkom and Davis, 1994; Baka et al., 1995). Cryopreservation may have adverse downstream consequences if the normality of embryogenesis is influenced by molecular and cellular activities in the oocyte that are labile to damage or disruption during the freezing–thawing process (Menéz and Guerin, 2004). For example, cellular alterations associated with osmotic forces produced during oocyte dehydration–rehydration cycles may have differential effects on cytoplasmic activities including mitochondrial metabolism and intracellular signalling pathways, such as those mediated by Ca2+. 

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1861
In previous studies (Van Blerkom et al., 2002, 2003), we suggested that high-polarized pericortical mitochondria in oocytes and early embryos may be involved in the modulation of focal levels of intracellular free Ca$^{2+}$and ATP. Here, mitochondrial polarity, ATP generation, and levels of intracellular free Ca$^{2+}$ were examined in thawed MII human oocytes that had been cryopreserved with a standard protocol used for oocytes and pronuclear/cleavage stage embryos.

**Materials and methods**

According to protocol, oocytes donated to research were obtained under the following conditions: (i) oocytes inseminated by conventional IVF or ICSI that failed to exhibit signs of fertilization 24 h after ovum retrieval (termed day 1: characterized by the absence of a second polar body or detectable pronuclei), (ii) unisemminated immature oocytes that matured to MII in vitro (Sage Fertilization Medium, Sage BioPharma, USA) and (iii) MII oocytes obtained exclusively for research and cryopreserved 3–4 h after retrieval (termed, day 0). All MII oocytes used in this study were denuded of cumulus and coronal cells by repeated passage though a glass micropipette, appeared grossly normal at the level of the dissecting microscope and at high magnifications, exhibited a cytoplasm that was uniform in texture and devoid of the types of dysmorphisms described previously (Van Blerkom, 1994).

**Cryopreservation–thawing**

Denuded oocytes were dehydrated for 20 min at room temperature in HEPE-buffered human tubal fluid (HTF) containing 1.5 mol/l 1,2-propanediol (PROH) and 12% human serum albumin (HSA) followed by an additional 10 min in a second solution containing 1.5 mol/l PROH and 0.2 mol/l sucrose (Tucker et al., 1996). At the end of the dehydration phase, oocytes were transferred to plastic cryostraws and cooled at a control rate of 2°C/min from room temperature to −7°C, at which point manual seeding was performed. After seeding, samples were cooled to −36°C at −0.3°C/min and then plunged directly into liquid nitrogen. Oocytes were thawed by exposing straws to air for 30 s followed by immersion in a water-bath for 45 s at 31°C. PROH was removed in two steps by passage of oocytes in 1 ml of HSA-supplemented HEPE-buffered HTF containing 0.5 mol/l (10 min) and 0.2 mol/l sucrose (10 min) respectively. Intact oocytes were transferred to normal embryo culture medium (Sage Cleavage medium, Sage BioPharma) supplemented with 10% HSA and pre-equilibrated in an atmosphere containing 5% CO$_2$ in air.

**Analysis of mitochondrial polarity (ΔΨm) with JC-1**

Human oocytes were exposed to inner mitochondrial membrane potential reporter JC-1 (5,5'-6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes, USA) for 30 min at 1 μg/ml in culture medium maintained at 37°C as previously described (Van Blerkom et al., 2002, 2003). Unfertilized and in vitro-matured oocytes were stained at 24 or 30 h post retrieval. Cryopreserved oocytes were stained at 0, 3 and 6 h post-thaw. The specificity of JC-1 staining of mitochondria was examined in randomly selected, representative oocytes by exposure to the proton ionophore FCCP [carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone; Sigma, USA] for 10 min at a concentration of 5 μmol/l prior to JC-1 exposure (Van Blerkom et al., 2002).

**Cortical granule staining**

Cortical granule staining followed protocols previously described for the human MII oocyte (Van Blerkom and Davis, 1994). Briefly, oocytes were exposed to an acidic Tyrode’s solution to remove the zona pellucida and were immediately fixed in phosphate-buffered saline (PBS) containing 3.7% formaldehyde for 30 min. After fixation, oocytes were subjected to three sequential 10 min washes in PBS followed by a 30 min incubation in modified PBS containing 5% bovine serum albumin (BSA), 0.1 mol/l glycine and 0.1% Triton X-100. Fixed and washed oocytes were exposed to biotinylated Lens culinaris lectin (LCA; 5 μg/ml) for 30 min, washed as above for 1 h, and stained with Texas Red–streptavidin (2 μg/ml) in modified PBS for 30 min followed by a 12–14 h incubation in modified PBS at 4°C. Oocytes were placed on glass coverslips in droplets of Slow-fade anti-quenching solution (Molecular Probes, USA) and examined by scanning laser confocal microscopy (SLCM).

**Microscopic analysis of JC-1 fluorescence**

JC-1-stained oocytes were maintained in modified HTF under oil at 37°C and examined by epifluorescence microscopy in the fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RTIC) channels using narrow band path filter sets (Van Blerkom et al., 2002).

**Estimation of free Ca$^{2+}$**

Estimation of free Ca$^{2+}$ levels by SLCM measured the average maximum relative fluorescence intensity (RFI) in individual control and thawed oocytes using the same analytical protocol described for the MII mouse oocyte (Van Blerkom et al., 2003). Briefly, oocytes were preloaded for 60 min in HEPE-buffered HTF supplemented with 4% BSA and 20 μmol/l Fluo-4 AM (Molecular Probes), followed by a 30 min wash in normal medium. Oocytes were transferred to ΔT dishes containing 1 ml of serum-free HEPE-buffered medium on an inverted microscope with temperature maintained at precisely at 37°C by means of a ΔT controller (Bioptics, USA). Five micrometre scans taken though the approximate centre of the oocyte established baseline RFI levels. Quantification of RFI used a long path filter with emission detection >510 nm and processing of the digital images with ImageSpace software (v3.10; Molecular Dynamics, USA). Specimens were examined at the same photomultiplier detector gain and laser intensity at 5 s intervals for up to 150 s after the addition of the Ca$^{2+}$ ionophore A23187 at 10 μmol/l as previously described (Van Blerkom et al., 2003).

**Measurement of net cytoplasmic ATP content**

Measurements of net ATP content followed a previously described protocol (Van Blerkom et al., 1995). Briefly, individual oocytes were rapidly frozen to −80°C in 200 ml of ultrapure water. ATP levels were quantified by measuring the luminescence (Berthold LB 9501 luminometer) generated in an ATP-dependent luciferin-luciferase bioluminescence assay (Bioluminescence Somatic Cell Assay System; Sigma, USA).

1862
A standard curve containing 14 ATP concentrations from 5 fmol to 5 pmol was generated for each series of analyses.

**Effects of cryoprotectants**

Representative oocytes were dehydrated and rehydrated as described above but without cryopreservation. After equilibration in normal embryo culture medium (10 min), oocytes were stained with JC-1 and examined by fluorescence microscopy.

**Statistical analysis**

The occurrence of J-aggregate fluorescence and differences in levels of intracellular free Ca\(^{2+}\) in ionophore-exposed fresh and thawed oocytes were analysed by Fisher’s exact test and unpaired t-test respectively.

**Results**

**Patterns of JC-1, J-aggregate fluorescence in human oocytes**

*Fresh MII stage oocytes*

A total of 166 unfertilized day 1 oocytes were obtained from 76 women between 29 and 38 years of age undergoing conventional IVF \((n = 44)\) or ICSI \((n = 32)\). Seventy-four per cent \((123/166)\) of unfertilized oocytes inseminated by conventional IVF \((n = 85)\) or ICSI \((n = 81)\) showed a pattern of punctate and circumferential pericortical J-aggregate fluorescence \((Figure 1B, C)\) identical to the one previously reported by \((Van Blerkom et al., 2002)\). Twenty-six per cent \((43/166)\) showed scant \((n = 12)\; white\; arrows, \(Figure 1D)\; combined\; fluorescence\; from\; FITC\; and\; RITC\; channels)\; or\; no \((n = 31)\) detectable pericortical J-aggregate fluorescence. Similar to findings described by \(Van Blerkom et al.\; (2002)\), the occurrence of J-aggregates in these cells indicates that their mitochondria were apparently unaffected by cryopreservation \((Figure 1K)\), and that under the present conditions, JC-1 staining was normal. In a second series of experiments, intact day 0 \((n = 4)\) and day 1 \((n = 15)\) thawed MII oocytes were stained with JC-1 and cultured for 4 days. All 19 oocytes remained intact at the end of this culture period.

In a third series of experiments, fresh GV \((n = 13)\), MI \((n = 5)\) and MII \((n = 10)\) oocytes from fertile ovum donors \((day\; 0)\) were stained \((at\; 37\; ^\circ\; C)\; with\; JC-1\; during\; or\; after\; dehydration. Approximately 90% \((25/28)\) of these oocytes showed normal pericortical J-aggregate fluorescence \((similar\; to\; images\; in\; Figure 1B\; and\; C)\) indicating that exposure to cryoprotectants alone, under the conditions used in this study, does not appear to depolarize mitochondria.

**Cortical granule staining**

Twenty-six thawed MII oocytes obtained from day 0 \((n = 14)\) and day 1 groups \((all\; ICSI-failed\; fertilization;\; n = 12)\) were stained to resolve cortical granules by SLCM. While morphometric assessments were beyond the scope of this study, all day 0 and 75% \((9/12)\) of day 1 oocytes exhibited intense circumferential cortical granule staining comparable in density and distribution to fresh oocytes \((Figure 1L; \; Table 1)\). Thee day 1 ICSI oocytes displayed cortical granule staining but at reduced intensity \((images\; not\; shown)\).

**Intracellular free calcium and ATP levels**

Owing to the limited availability of oocytes, the following preliminary studies were conducted to determine ATP contents or levels of intracellular free Ca\(^{2+}\). Changes in the RFI described \((Van Blerkom et al., 2002)\), the punctate fluorescence indicated by black arrows in Figure 1D, E, H and I is autofluorescence associated with small lipid-containing inclusions.

**Cryopreserved MII oocytes**

Approximately 40% \((14/36)\) of day 0 and 62% \((50/80)\) of day 1 oocytes were intact after rehydration \((Figure 1H)\). Day 0 oocytes were stained at timed intervals after rehydration \((0\; h,\; n = 8;\; 3\; h,\; n = 3;\; 6\; h,\; n = 3)\). A similar protocol was used for day 1 oocytes at 0 h \((n = 28)\), 3 h \((n = 15)\) and 6 h \((n = 7)\). All day 0 and day 1 oocytes were positive for JC-1 uptake \((Figure 1G)\). None of the day 0 oocytes showed detectable pericortical J-aggregate fluorescence. Seventeen per cent \((9/50)\) of day 1 oocytes showed J-aggregate fluorescence, but the circumferential signal was either of a reduced intensity \((similar\; to\; image\; shown\; in\; Figure 1D)\) when compared to their fresh counterparts or was localized to one region of the pericortical cytoplasm \((Figure 1D)\). Differences between J-aggregate positive fresh and J-aggregate negative thawed oocytes were significant \((Table I)\). In all instances where some patches of cumulus and corona cells remained adherent to the zona pellucida after thawing, definitive J-aggregate fluorescence occurred in these cells. As previously described for fresh oocytes \((Van Blerkom et al., 2002)\), the occurrence of J-aggregates in these cells indicates that their mitochondria were apparently unaffected by cryopreservation \((Figure 1K)\), and that under the present conditions, JC-1 staining was normal. In a second series of experiments, intact day 0 \((n = 4)\) and day 1 \((n = 15)\) thawed MII oocytes were stained with JC-1 and cultured for 4 days. All 19 oocytes remained intact at the end of this culture period.
of Fluo-4-stained oocytes exposed to A23187 were examined in fresh day 1 oocytes \((n = 15)\) and in thawed oocytes, randomly selected from day 0 \((n = 11)\) and day 1 groups \((n = 9)\). The maximum average increase (untreated, Figure 1M) in RFI above background in fresh oocytes was \(\geq 400\%\) (80 s after the addition of ionophore, Figure 1N), while for thawed human oocytes (untreated, Figure 1O) the maximum average increase at 90 s after ionophore treatment was \(\approx 50\%\) (Figure 1P), and these differences were significant \((P < 0.01)\) (Table I). RFI was determined from pseudocolour images where the increase in the intensity of Fluo-4 fluorescence was measured on a scale from 0 to 255 (colour bar, Figure 1N).

We did not determine whether the oocytes used for Fluo-4 analysis were J-aggregate positive or negative owing to mutual fluorescence of Fluo-4 and the JC-1 monomer in the FITC channel. However, as noted above, all thawed day 0 and most day 1 oocytes were J-aggregate negative.

In a second series of analyses, the net ATP content of individual day 1 oocytes was determined after thawing and culture (6 h). The average ATP contents of J-aggregate positive \((n = 17; 1.96 \text{ pmol/oocyte} \pm 200 \text{ fmol})\) and negative \((n = 29; 1.91 \text{ pmol/oocyte} \pm 200 \text{fmol})\) oocytes were not significantly different and were comparable to values previously reported for fresh MII oocytes (Van Blerkom et al., 1995).
Table 1. Mitochondrial polarization, cortical granules and up-regulation of intracellular Ca\(^{2+}\) in fresh and thawed metaphase II human oocytes

<table>
<thead>
<tr>
<th>Oocyte category</th>
<th>J-aggregate positive % (n)</th>
<th>Cortical granule positive % (n)</th>
<th>Average increase in Fluo-4 RFI units after A23187 exposure % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>92 (68)(^{a})</td>
<td>100 (39)</td>
<td>n/a</td>
</tr>
<tr>
<td>Frozen</td>
<td>0 (14)(^{a})</td>
<td>100 (14)</td>
<td>22 (11)(^{b})</td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>77 (275)(^{c})</td>
<td>80 (12)</td>
<td>117 (15)(^{d})</td>
</tr>
<tr>
<td>Frozen</td>
<td>17 (50)(^{c})</td>
<td>75 (12)</td>
<td>26 (9)(^{c,d})</td>
</tr>
</tbody>
</table>

\(^{a}\)Fisher’s exact test: \(P < 0.0001\).
\(^{b}\)Unpaired t-test: \(P = 0.0002\).
\(^{c}\)Fisher’s exact test: \(P < 0.0001\).
\(^{d}\)Unpaired t-test: \(P = 0.01\).

n/a = not applicable.

Discussion

Mitochondrial respiration is the primary source of ATP in the mature human oocyte and early embryo, and several recent studies have used the fluorescent \(\Delta\Psi_m\) reporter JC-1 to determine whether a relationship exists between developmental competence and metabolic activity (Ahn et al., 2002; Wilding et al., 2002; Van Blerkom et al., 2003). The apparent state of polarization detected with JC-1 is usually thought to reflect differences in mitochondrial respiratory activity and Ca\(^{2+}\) sequestration, with highest levels presumably associated with hyperpolarized mitochondria that fluoresce red owing to the formation of J-aggregates, while their lower polarized counterparts fluoresce green (for details on the properties of this probe see: Reers et al., 1995).

We report that cryopreservation had two negative consequences for the oocyte: (i) loss of the ability of pericortical mitochondria to retain high polarity and form J-aggregates and (ii) a significant diminution in the ability of the thawed oocyte to up-regulate Ca\(^{2+}\) in response to the Ca\(^{2+}\) ionophore A23187. The extent to which loss of high polarity contributed to the diminished ability to up-regulate Ca\(^{2+}\) is unknown, and the present studies do not preclude the possibility that cryopreservation-associated defects or damage to other Ca\(^{2+}\)-regulating elements that act in concert with mitochondria, such as the smooth endoplasmic reticulum (SER), may be involved (Hajnoczky et al., 2000; Liu et al., 2001). However, it is an intriguing possibility that the relationship between polarization, mitochondrial respiration and the regulation of intracellular free Ca\(^{2+}\) described for somatic cells (Ichas et al., 1997; Smaili and Russell, 1999) and mouse oocytes (Liu et al., 2001; Dumollard et al., 2003; Van Blerkom et al., 2003) also pertains to the human female gamete.

Ozil and Huneau (2001) reported that a reduction in the amplitude of the first Ca\(^{2+}\) transient during the first seconds of oocyte activation was associated with growth retardation and developmental anomalies in rabbit embryos that were not evident until day 11.5 of embryogenesis. In the human MII oocyte, the pericortical distribution of J-aggregate fluorescence is associated with clusters of mitochondria that surround or are embedded within discrete, spheroidal SER networks (Van Blerkom et al., 2002). The possibility that some proportion of embryos derived from thawed oocytes are unable to maintain normal Ca\(^{2+}\) signalling owing to cryopreservation-induced defects in mitochondrial polarity warrants further investigation. In this respect, irreversible mitochondrial depolarization coupled with sustained high levels of intracellular free Ca\(^{2+}\) and diminished ATP production are cellular events leading to activation of the apoptotic pathway (Kroemer and Reed, 1997; Duchen, 2000). However, loss of mitochondrial hyperpolarization does not appear to be associated with apoptosis in thawed human oocytes because: (i) they exhibit a diminished capacity to increase intracellular Ca\(^{2+}\), (ii) they show levels of ATP comparable to fresh oocytes, (iii) they are fertilizable by ICSI and capable of significant \textit{in vitro} development, and (iv) they remained intact after 4 days of culture in the present study.

The ability of pericortical mitochondria to form J-aggregates during dehydration indicates that high polarity may be lost during the slow cool stages that precede or follow seeding. The detection of an apparently normal level of cortical granules thoughout the circumference of J-aggregate negative thawed oocytes suggests that if Ca\(^{2+}\) is released during this stage, it is either below levels that can induce cortical granule exocytosis or that their exocytosis is inhibited at reduced temperatures. In the short term, loss of high \(\Delta\Psi_m\) did not appear to compromise the ability of the thawed human oocyte to generate ATP at levels comparable to their fresh counterparts. Therefore, the relationship between \(\Delta\Psi_m\) and respiratory activity in fresh and thawed human oocytes needs further investigation that includes kinetic analyses of ATP turnover. For the human, one possible explanation for compromised competence currently under investigation is that after thawing, the inability of mitochondria to hyperpolarize may have downstream effects on respiratory capacity during stages of development where higher demands for ATP may exist, especially if persistent hypopolarization makes mitochondria refractory to changes in ambient Ca\(^{2+}\) that could up-regulate respiration.

Support for a mitochondrial polarity association with competence would be indicated if the relatively small proportion of thawed oocytes that retained the ability to form pericortical J-aggregates are shown to be responsible for successful outcomes, or if new protocols of oocyte cryopreservation prevent the apparent irreversible changes in mitochondrial polarity we describe. The present results confirm our earlier report that a variable proportion of fresh MII oocytes obtained from infertile women are J-aggregate negative (Van Blerkom et al., 2002). These preliminary findings suggest the possibility that maintenance of high polarized mitochondria may be related to oocyte competence, and, as such, it may be useful to determine whether loss of polarity is associated with certain aetiologies of infertility or outcomes in IVF cycles. If an association between J-aggregate fluorescence and human oocyte competence exists, as suggested for the early human embryo (Wilding et al., 2003), the finding that some proportion of fresh MII oocytes \textit{(in vivo- and \textit{in vitro}-matured)} are J-aggregate negative may...
need to be considered when oocyte cryopreservation is contemplated.

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