The effect of chilling on membrane lipid phase transition in human oocytes and zygotes

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BACKGROUND: Chilling injury occurs when the cell membrane undergoes a transition from the liquid state to the gel state. Human oocytes and single-cell zygotes are of similar shape and size but the post-thawing survival rate of oocytes is poorer. We set out to investigate the possible difference in membrane lipid phase transition (LPT) temperature between the two cell types. METHODS: The LPT temperature was measured with a Fourier Transform Infra-red analyser, which detects the change in the vibration frequency of the CH₂ bond stretches of the membrane lipid molecules during temperature change. The LPT temperatures of unfertilized human oocytes, in vitro-matured oocytes, and immature germinal vesicle (GV) stage oocytes were compared with that of abnormally fertilized human zygotes. RESULTS: The LPT temperatures of zygotes and of mature and immature GV oocytes differ significantly from each other (10.0 ± 1.2, 16.9 ± 0.9 and 24.4 ± 1.6°C respectively; P < 0.05). CONCLUSIONS: Zygotes show a higher resistance to chilling injury compared to oocytes at different developmental stages; this might explain the relatively poor survival rates of cryopreserved human oocytes and indicates the necessity to adjust the cryopreservation protocols in order to minimize cryoinjury.

Key words: chilling injury/cryopreservation/human oocyte/human zygote/lipid phase transition

Introduction

Cryopreservation of human sperm cells and embryos has been successfully applied for several years and contributes to the increasing success of assisted reproduction technologies. Unfortunately, despite years of research, cryopreservation of human oocytes still yields unsatisfactory results and is considered experimental. The ability to cryopreserve oocytes would be beneficial in overcoming moral, ethical and legal issues that arise from embryo cryopreservation. It might also offer the prospect of preserving the fertility of women scheduled for chemo or radiation therapy.

During the process of cryopreservation, cells are exposed to mechanical, thermal and chemical stresses. Oocyte freezing is accompanied by various types of injury, including damage to the meiotic spindle and to the microfilaments (Pickering et al., 1990; Baka et al., 1995; Park et al., 2000; Boiso et al., 2002; Mullen et al., 2004; Bianchi et al., 2005) and zona pellucida hardening. Due to injury inflicted during cryopreservation, survival rate is low as reviewed by Van der Elst (2003).

There are two main steps in the process of cryopreservation: (i) chilling, i.e. lowering the temperature from the physiological temperature to the freezing point; and (ii) freezing, further reducing the temperature to the storage temperature (liquid nitrogen at −196°C). Chilling injury is defined as permanent damage that occurs upon cooling to low but not freezing temperatures. Chilling injury can modify the structure of membranes and, therefore, their integrity (Arav et al., 1996; Zeron et al., 1999). Phospholipids are major components of cellular membranes; the length of the fatty acid acyl chain, the number and positions of the double bonds within the phospholipids strongly influence the membrane properties (Stubbs and Smith, 1984; Arav et al., 2000a). High concentrations of cholesterol and polyunsaturated fatty acids (PUFA) (Quinn, 1985; White, 1993) render the membrane more fluid at low temperatures and decrease its sensitivity to chilling injury. The membrane lipid composition is used as an indicator of changes in membrane fluidity, which depends on the cholesterol level, the phospholipid composition, the degree of unsaturation and the protein content (Giraud et al., 2000). As cooling results in removal of thermal energy, the molecular motion in the membrane lipid bilayer decreases, allowing interaction between adjacent lipid molecules, thus changing the fluidity and hence the functioning of the membrane. The temperature at which the membrane phospholipids transform from a liquid crystalline phase (Lα), characterized by high rotational and lateral mobility of the lipids and therefore by a high degree of fluidity, to a crystal gel phase (Lβ⁺) in which the mobility of the lipids is restricted, is called the ‘transition temperature’. The centre of the lipid phase transition (LPT)
curve—the point of transition from \( L\alpha \) to \( L\beta \) is designated \( T_m \) and is determined by statistical analysis (Crowe et al., 1989).

The susceptibility of oocytes to chilling injury had been reported elsewhere (Didion et al., 1990; Parks and Ruffing, 1992; Arav et al., 1993; Otoi et al., 1995), and it had been suggested that chilling injury also affects the oocyte microtubules (Albertini and Eppig, 1995), the cytoskeletal organization (Overstrom et al., 1990) and the zona pellucida (Vajta et al., 1998). LPT was associated with direct chilling injury in pig sperm membranes (Drobnis et al., 1993) and in bovine (Arav et al., 1996), sheep (Zeron et al., 2002a) and zebra fish (Pearl and Arav, 2000) oocytes.

It was demonstrated that a low LPT was associated with chilling resistance (Arav et al., 1993), therefore we decided to evaluate the LPT of relatively highly resistant single-cell human zygotes that had been successfully cryopreserved in IVF programmes. The relatively poor survival rate of frozen–thawed oocytes compared with that of single-cell 2-pronuclei (PN) zygotes that are similar in shape and size, led us to investigate whether there is a difference in LPT temperature between human oocytes and zygotes.

Materials and methods

Biological material

Oocytes included in this study were donated by consenting patients undergoing IVF/ICSI procedures and represent material that is otherwise discarded. The study was approved by the Local Review Board.

The ovulation induction protocol (Shulman et al., 1996) and the ICSI procedure (Van Steirteghem et al., 1993) were as described previously. Sperm-injected oocytes that had failed to be fertilized, or immature oocytes not suitable for ICSI were used (Ghetler et al., 1998). Metaphase I oocytes were matured in vitro for 24 h in Cook Culture System (Cook, Brisbane, Australia) until they reached the metaphase II (MII) stage. They were analysed, together with the unfertilized oocytes, 24 h after retrieval, whereas the germinal vesicle (GV) oocytes were analysed on the retrieval day. The zygotes used in the present study were abnormally fertilized oocytes that exhibited one or three pronuclei 16–20 h after ICSI. According to the ICSI procedure, all oocytes were free of cumulus cells after treatment with hyaluronidase (Cook).

Evaluation of membrane phase transition

Each analysed oocyte or zygote was individually placed between two quartz slides separated by a thin layer of silicone grease and compressed until the specimen was flattened to a thin layer of cytoplasm. Care was taken to keep the membrane intact. The quartz slides containing the sample were loaded into a temperature controller and placed on the microscope stage. The temperature was regulated by a microprocessor feedback system that measured the sample temperature with a thermocouple attached to the glass. The temperature was adjusted to the desired level and allowed to equilibrate before the specimen was scanned. Scanning was performed at the range of 28 to 2, at 2°C intervals. A total of 66 scans at a resolution of 4 cm\(^{-1}\) were collected for each sample at each temperature. The evaluation was conducted with a Bruker Equinox 55 Fourier Transform Infrared (FTIR) analyser connected to a Bruker A590 FTIR microscope equipped with a liquid nitrogen-cooled mercury cadmium telluride detector (Arav et al., 1996, 2000a; Zeron et al., 2002a). The \( T_m \) values were calculated from the frequency–temperature plots by statistical analysis as described by Crowe et al. (1989).

Statistics

The significance of differences between experimental groups was determined by one-way analysis of variance; \( P < 0.05 \) was considered significant.

Results

FTIR spectroscopy measures the vibrational frequency of methylene (\( \text{CH}_2 \)) groups, most of which are in the hydrocarbon chain of lipid molecules. The membrane phase transition was detected by monitoring the change in the vibration frequency of the \( \text{CH}_2 \) bond stretches as the temperature changed. The peak showed a shift in wave number when the lipid underwent a phase transition. Figure 1 shows a schematic illustration of the wave shift following the change in \( \text{CH}_2 \) frequency of the membrane lipids (change from liquid phase \( L\alpha \) to gel phase \( L\beta \)), with change in temperature.

The evaluated value of \( T_m \) differed among the groups examined. As demonstrated by Crowe et al. (1999), the changes in FTIR frequency in the isolated membranes were in excellent agreement with the data they obtained with differential scanning calorimetry, indicating that our FTIR results are reliable. Furthermore, when the plasma membrane and the internal membranes were analysed separately, Crowe et al. (1999) recorded similar transitions.

Oocytes

Immature oocytes

The \( T_m \) of GV oocytes (\( n = 6 \)) was 24.4 ± 1.6°C (mean ± SE) (Figure 2a).

![Figure 1. Schematic illustration of the wave shift following the change in \( \text{CH}_2 \) frequency of the membrane lipids (change from liquid phase \( L\alpha \) to gel phase \( L\beta \)) with change in temperature.](image-url)
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Mature oocytes

When the unfertilized oocytes (n = 13) and the in vitro-matured MII oocytes (n = 7) were analysed separately we found no significant difference (P > 0.05) between their $T_m$ values (15.7 ± 3.2 and 18.9 ± 1.3°C respectively), therefore they were pooled into one group of mature oocytes.

The phase transition temperature of this group of mature oocytes (n = 20) was 16.86 ± 0.86°C (Figure 2b).

Zygotes

The LPT of single-cell zygotes (n = 10) occurred at a significantly lower temperature than that of oocytes, i.e. at 10.04 ± 1.22°C (Figure 2c).

Statistical evaluation of the measured data showed significant differences between all the study groups (Table I).

Discussion

During cryopreservation, cells are exposed to low, non-physiological temperatures and thus become vulnerable to chilling injury because of the thermotropic LPT. The lipid composition of the membrane strongly influences its properties, including its resistance to thermal stress (Arav et al., 2000a; Zeron et al., 2001, 2002b).

The chilling injury is temperature dependent; it is caused by changes in the membrane properties and integrity and is responsible for the extensive cell damage that occurs during the process of cryopreservation. We examined, for the first time, the LPT of human mature oocytes and human zygotes, and our findings matched the preliminary results reported by Reuvenof-Pass et al. (2003) regarding the $T_m$ of GV oocytes. Since the availability of MII human oocytes donated for research is scarce and sporadic, the ‘failed to fertilize’ and ‘in vitro-matured’ oocytes were chosen as the closest alternative for mature oocytes. These oocytes were incubated at the same culture conditions and culture medium as the tested human zygotes.

Human zygotes, used in the current research, presented an abnormal number of PN after ICSI treatment. These fertilized oocytes failed to either form the male PN (1PN), or to extrude the 2nd polar body (3PN). These zygotes can, however, represent a normal zygote (2PN) since the developmental abnormality is not due to inadequate cortical granule exocytosis (Ghetler et al., 1998) and their membranes, similar to normally fertilized oocytes, were exposed to cortical granule exudates.

Since the oocytes and zygotes were free of surrounding cumulus cells and were tightly compressed between the two quartz slides, the analysed section contained mostly the analysed cell membrane and a minimal amount of cytoplasm (Arav et al., 1996; Crowe et al., 1999).

**Table I.** Lipid phase transition temperatures ($T_m$) of human immature germinal vesicle (GV) oocytes, mature oocytes and zygotes of the study groups

<table>
<thead>
<tr>
<th>Study group</th>
<th>n</th>
<th>$T_m$ (°C) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature (GV) oocytes</td>
<td>6</td>
<td>24.4 ± 1.6a</td>
</tr>
<tr>
<td>Mature oocytes</td>
<td>20</td>
<td>16.9 ± 0.9b</td>
</tr>
<tr>
<td>Zygotes</td>
<td>10</td>
<td>10.0 ± 1.2c</td>
</tr>
</tbody>
</table>

*a*Values represent (mean ± SE).

*b, c*Different superscripts within the column denote significant differences (P < 0.05).

![Figure 2](image-url)
Our results clearly demonstrate a significant difference between the phase transition temperatures in zygotes and oocytes (at different developmental stages). Cryopreservation of zygotes is widely used as a routine procedure in IVF treatment, and it yields satisfactory results (Al-Hasani et al., 1996). The $T_m$ of human zygotes is low, indicating a relatively good resistance of the membrane to chilling injury, nevertheless the $T_m$ of human oocytes is significantly higher than that of zygotes, which suggests a greater sensitivity to chilling. In the present study the most sensitive group was the GV oocytes. Arav et al. (1996) also found that the membrane of GV-stage bovine oocytes was much more susceptible to cryoinjury than that of mature oocytes. It became more tolerant to chilling after electrofusion with large fragments from membranes of mature oocytes, indicating that alteration of the membrane composition could change its thermal behaviour and consequently its chilling resistance. The changes in the thermal behaviour of oocyte membranes at different developmental stages might suggest that there are changes in the phospholipid composition and the cholesterol concentration.

The slow-freezing, rapid-thawing protocol, with 1,2-propanediol cryoprotectant, that is routinely used for cryopreservation of human zygotes, involves exposure to cryoprotectants at room temperature, prior to the freezing process, which starts at $20^\circ$C. This protocol is suitable for zygote cryopreservation, since the phase transition temperature of zygotes is far below $20^\circ$C, and the protocol is indeed successfully employed in IVF treatments. In contrast, the $T_m$ of mature oocytes is very close to room temperature, at which a part of the procedure takes place, and that of GV oocytes is above this temperature, which renders their membranes prone to chilling injury. The high LPT temperature may explain, at least in part, the poor survival rate of human female gametes under cryopreservation. Lim et al. (1991) demonstrated that bovine single-cell zygotes have higher post-thaw survival rates and better developmental capacities than those of MII oocytes. Furthermore, the survival, fertilization and developmental rates of MII oocytes after slow freezing were better than those of GV oocytes. Also for mouse oocytes, higher cryosensitivity was found in immature than in mature oocytes (Schroeder et al., 1990; Candy et al., 1994). We have demonstrated in our present study that the $T_m$ of human zygotes is low. Since biological processes are very slow at low temperatures the kinetics of injury are slow as well, which enables the critical $T_m$ to be passed with relatively minor damage. The high $T_m$ values of MII and GV oocytes might be the underlying cause of the extensive cryoinjury to membranes.

Alternative methods for cryopreservation of human oocytes rely on passing through the transition phase temperature rapidly, and so partially avoiding the induction of membrane cryoinjury by the LPT. Among these methods are the vitrification method (Hong et al., 1999; Arav et al., 2000b; Cha et al., 2000; Yoon et al., 2000; Jelinkova et al., 2002), and the rapid vitrification method with accelerated cooling. Accelerated cooling is achieved by using open pooled straws (Vajta et al., 1998), electron microscope grids (Martino et al., 1996; Park et al., 2000), minimal drop size (Arav and Zeron, 1997; Yavin and Arav, 2001) or supercooled liquid nitrogen (Arav et al., 2002). Changing the handling temperature to $37^\circ$C was reported to improve the outcome of cryopreservation in mouse GV oocytes (Isachenko and Nayudu, 1999).

Artificial alteration of the lipid membrane composition, e.g. by incorporation of liposomes into the membrane (Zeron et al., 2002b), can create chilling-resistant oocytes. This method can be used to improve the outcome of oocyte cryopreservation and also to contribute to the development of better cryobanking programs. Enriching ewes’ diets with polyunsaturated fatty acid (PUFA) (Zeron et al., 2002a) demonstrated changes in the fatty acid profile of the oocytes. These oocytes exhibited changes in the physical properties of their membranes and a decrease in the lipid transition temperature from 15 to 4$^\circ$C, which indicates enhanced resistance to chilling injury.

Membranes contain a variety of phospholipids, all of which affect the $T_m$. The fact that membranes of zygotes and of oocytes at different developmental stages have different $T_m$ values, suggests a meaningful difference between their compositions, the $T_m$ being strongly related to the PUFA content (Zeron et al., 2002a). Matorras et al. (1998) demonstrated that mature human oocytes contain mainly (79.22%) saturated fatty acids and only 6.5% PUFA. Certain changes in membrane composition might occur during oocyte maturation from the GV to the MII stage, and still greater changes after fertilization. We can speculate that one of the reasons for this change in zygotes might be the contribution of exocytosis of cortical granules that occurs at fertilization. More information about the composition of the membranes of human zygotes and of human oocytes at different developmental stages is needed.

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