Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws

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Vitrification of oocytes has been applied recently for humans, but remains elusive. The microtubules of oocytes are vulnerable to cryoprotectants and thermal changes. Using mouse oocytes, the effects of vitrification in open pulled straws (OPS) were investigated on survival, the meiotic spindle, and chromosomes and compared with conventional straws. Mature oocytes were allocated to four groups for exposure to cryoprotectants, vitrification in conventional straws, or vitrification in OPS. They were diluted in stepwise sucrose solutions. Oocytes without treatments were used as controls. The surviving oocytes were stained for meiotic spindles and chromosomes. After dilution, all of the oocytes exposed to cryoprotectants survived. Vitrification sometimes resulted in lysis so that survival using OPS (62%) was significantly \( P < 0.05 \) smaller than that using conventional straws (81%). Oocytes exposed to cryoprotectants or vitrified exhibited serious disturbances of microtubules immediately post-dilution. After 1 h incubation, the microtubules could repolymerize so that the OPS group had significantly \( P < 0.05 \) more normal spindles (78%) than did the conventional straw group (21%). The former also tended to have more compact chromosomes (87%) than did the latter (78%). OPS for vitrification of oocytes achieve more rapid cooling, warming, and dilution and so reduce spindle injury. However, the lower survival rate in OPS needs improvement.

Key words: conventional straws/meiotic spindle/oocyte vitrification/open pulled straws

Introduction

The cryopreservation of human oocytes would make a significant contribution to infertility treatments. It could also provide an alternative to embryo preservation for the avoidance of ethical problems. Embryo cryopreservation is now a successful procedure, but oocyte cryopreservation has poorer results (Bernard and Fuller, 1996; Mandelbaum et al., 1998). Only a few successful pregnancies have arisen from cryopreserved human oocytes (Chen, 1986; Porcu et al., 1998; Tucker et al., 1998). This situation has been primarily attributed to the poor survival, fertilization, and development of cryopreserved human oocytes (Trounson and Kirby, 1989; Van Blerkom and Davis, 1994).

The majority of pregnancies from cryopreserved oocytes in humans were achieved by the slow freezing method (Chen, 1986; Porcu et al., 1998; Tucker et al., 1998). However, the viability of oocytes after thawing has been highly variable, ranging from 2.2 to 64.0%, and deserves further refinement (Gook et al., 1993; Tucker et al., 1998). The vitrification method for cryopreservation of oocytes seems promising according to results from mammalian experiments (Nakagata, 1989; Hotamisligil et al., 1996; Martino et al., 1996; Vajta et al., 1998). The value of vitrification for human oocytes remains elusive (Hunter et al., 1995), although successful case reports have been documented (Hong et al., 1999; Kuleshova et al., 1999).

Otoi et al. (1998) vitrified bovine oocytes that were very sensitive to chilling with ethylene glycol-based cryoprotectants in conventional straws, and they achieved results better than those observed with the slow freezing method. To obtain a more rapid cooling and warming rate, Vajta et al. (1998) developed open pulled straws (OPS) to hold bovine oocytes with a very small amount of solution for vitrification. They reported that the developmental capacity and the pregnancy potential of oocytes vitrified using OPS were improved, compared with those vitrified using conventional straws. The comparative merits of OPS and conventional straws for the vitrification of oocytes deserve further investigation.

The microtubules of oocytes are vulnerable to cryoprotectants and thermal changes involved in cryopreservation (Pickering et al., 1990; Van Blerkom and Davis, 1994). Disruption of the meiotic spindle may lead to impairment of fertilization of oocytes and the growth of embryos (Eroglu et al., 1998). Although the microtubular system of mouse oocytes with respect to the distribution of pericentriolar material is different from that of human oocytes, it has been widely used as a model to study the spindle organization of human oocytes (Pickering et al., 1990; Joly et al., 1992). Using this model, our study explored the effects of vitrification of oocytes using conventional straws and OPS on morphological survival, the meiotic spindles, and chromosomes.

Materials and methods

Preparation of oocytes

Female ICR mice aged 6-8 weeks were induced to superovulate by i.p. injection of 10 IU of pregnant mare’s serum gonadotrophin
Vitrification of oocytes in OPS

The oocytes were then washed, transferred into the culture medium, and incubated at 37°C.

**Vitrification of oocytes in conventional straws**

The oocytes (four to six at a time) were pretreated with 1.5 mol/l ethylene glycol (EG) for 5 min. They were transferred into a drop (200 µl) of EG5.5 on a Petri dish (Becton Dickinson and Company, Lincoln Park, NJ, USA) and mixed for equilibration. They were then placed on the other drop (200 µl), and the time for exposure to EG5.5 was 1 min. The procedures were performed at a room temperature of 22–24°C. Next, the oocytes were transferred into 0.5, 0.25 and 0.125 mol/l sucrose in a 4-well dish (Becton Dickinson and Company), for 2.5 min in each solution at 37°C. The oocytes were then washed, transferred into the culture medium, and incubated at 37°C.

**Definition of morphological survival**

Oocytes were defined as having morphologically survived if the cells possessed an intact zona pellucida and plasma membrane and refractive cytoplasm. They were counted and recorded.

**Fluorescent staining of meiotic spindles and chromosomes**

The morphologically surviving oocytes of the treatment groups were examined for spindles and chromosomes immediately post-dilution and after 1 h of incubation. The control oocytes were also examined at retrieval and after 1 h incubation for comparisons. Fixation and staining were adapted from a published method (Pickering et al., 1990). Preservation of oocytes was achieved by fixation in 2% formaldehyde (Merck, Darmstadt, Germany) with 0.02% Triton X-100 (Merck) in DPBS at 37°C for 30 min. The oocytes were then incubated in anti-α-tubulin monoclonal antibody (Sigma) in DPBS with 0.5% bovine serum albumin (BSA) for 45 min. They were then washed in 0.01% Tween-20 (Merck) for 15 min. Tubulin staining intensity was amplified by incubating the oocytes in fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma) for 45 min together with Hoechst 33258 (20 µg/ml) (Sigma) which stained the chromosomes. Excess antibody and dye were washed out in 0.01%...
The spindle morphology of the control oocytes at retrieval was barrel-shaped with microtubules traversing between both poles and chromosomes. Typically, the normal spindle morphology was barrel-shaped with microtubules, resulting in none having a normal spindle (Figure 2A). Oocytes exposed to cryoprotectants or vitrified either in conventional straws or in OPS had serious disturbances of the microtubular system, resulting in none having a normal spindle (Figure 2B). The conventional straw group tended to have greater percentages of oocytes with disrupted or absent spindles than the exposure group or the OPS group, but the differences were not significant.

After 1 h incubation, the spindle morphology of the control oocytes had not changed and most of them were normal (Table III). The injured spindles in the oocytes exposed or vitrified recovered to varying degrees after 1 h incubation post-dilution (Figure 2C,D). Oocytes exposed or vitrified either in conventional straws or in OPS had significantly smaller percentages of normal spindles than the controls. A significantly ($P < 0.05$) smaller percentage of normal spindles were restored in oocytes vitrified in conventional straws (21%) than in oocytes exposed (84%) and those vitrified in OPS (78%). The differences between oocytes exposed and those vitrified in OPS were not obvious. Reduced spindles made up the majority of abnormal patterns.

Chromosomal patterns of the control oocytes at retrieval and of those exposed or vitrified at immediate fixation post-dilution are shown in Table IV. Compact chromosomes were present in most of the control oocytes (98%). Oocytes exposed or vitrified either in conventional straws or in OPS had significantly smaller percentages of compact chromosomes and appeared to have greater chromosomal dispersion than the controls, but the latter difference was not significant between the three treatment groups.

With 1 h incubation, the chromosomal patterns of control oocytes did not alter in that 98% were compact. The incidences of chromosomal dispersion decreased after the 1 h incubation post-dilution in the groups of exposure or vitrification (Table V). In comparison with the controls, oocytes vitrified either in conventional straws or in OPS had significantly smaller ($P < 0.05$) percentages of compact chromosomes. The OPS group tended to have a greater percentage of compact chromosomes (87%) than the conventional straw group (78%) but the difference was not significant.

### Results

The morphological survival rates of oocytes in the three treatment groups were calculated. The patterns of the meiotic spindles and chromosomes immediately and after 1 h incubation were analysed for the four groups of oocytes. The $\chi^2$-test was used for statistical comparisons. $P < 0.05$ was considered significant.

### Discussion

The exposure of oocytes to EG5.5 and then their transfer to 0.5 mol/l sucrose with stepwise dilutions did not result in lysis. Without cooling, the oocytes could tolerate the osmotic changes from the concentrated cryoprotectants to dilution solutions. After vitrification with cooling, oocytes become less resistant to the osmotic stress and may suffer damage during rehydration (Hotamisligil et al., 1996). Vitrification of mammalian oocytes using conventional straws has been extensively studied and has achieved a high rate of morphological survival (Nakagata, 1989; Hotamisligil et al., 1996; Otoi et al., 1998). Vajta et al. (1998) recently developed the method of OPS for vitrification of bovine oocytes and reported a favourable capacity for development. However, the survival ability of
Table II. Spindle morphology of control oocytes at retrieval and of those exposed to cryoprotectants, vitrified in conventional straws, or vitrified in open pulled straws (OPS) at immediate fixation post-dilution

<table>
<thead>
<tr>
<th>Conditions</th>
<th>No. of oocytes</th>
<th>Spindle morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Control</td>
<td>59</td>
<td>56 (95)</td>
</tr>
<tr>
<td>Exposed</td>
<td>55</td>
<td>0 (0)b</td>
</tr>
<tr>
<td>Straws</td>
<td>63</td>
<td>0 (0)b</td>
</tr>
<tr>
<td>OPS</td>
<td>54</td>
<td>0 (0)c</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

a,b,c,p < 0.05, compared with control oocytes.

Figure 2. The patterns of the spindles and chromosomes of a control oocyte and three vitrified oocytes using the two different methods are shown. PB = area of polar body structures. (A) A control oocyte has a normal barrel-shaped spindle in the ooplasm. The metaphase chromosomes align regularly on the metaphase plate. (B) At immediate fixation post-dilution, an oocyte vitrified in a conventional straw discloses disruption of the spindle with dispersion of chromosomes. (C) After 1 h incubation post-dilution, an oocyte vitrified in a conventional straw demonstrates a reduced spindle and normal chromosomes. (D) After 1 h incubation post-dilution, an oocyte vitrified in open pulled straws recovers its normal spindle organization and compact chromosomes. Scale bar = 20 µm.

Table III. Spindle morphology of control oocytes following 1 h incubation and of those exposed to cryoprotectants, vitrified in conventional straws, and vitrified in open pulled straws (OPS) after 1 h incubation post-dilution

<table>
<thead>
<tr>
<th>Conditions</th>
<th>No. of oocytes</th>
<th>Spindle morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Control</td>
<td>57</td>
<td>55 (96)</td>
</tr>
<tr>
<td>Exposed</td>
<td>63</td>
<td>53 (84)a</td>
</tr>
<tr>
<td>Straws</td>
<td>67</td>
<td>14 (21)b,d</td>
</tr>
<tr>
<td>OPS</td>
<td>55</td>
<td>43 (78)c</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

a,b,c,p < 0.05, compared with control oocytes.

d,p < 0.05, compared with oocytes exposed to cryoprotectants or vitrified in OPS.
Table IV. Chromosomal patterns of control oocytes at retrieval and of those exposed to cryoprotectants, vitrified in conventional straws, and vitrified in open pulled straws (OPS) at immediate fixation post-dilution

<table>
<thead>
<tr>
<th>Conditions</th>
<th>No. of oocytes</th>
<th>Chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>59</td>
<td>58 (98)</td>
</tr>
<tr>
<td>Exposed</td>
<td>55</td>
<td>45 (81)(^a)</td>
</tr>
<tr>
<td>Straws</td>
<td>63</td>
<td>47 (75)(^b)</td>
</tr>
<tr>
<td>OPS</td>
<td>54</td>
<td>42 (78)(^c)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

\(^a,b,c\) P < 0.05, compared with control oocytes.

Table V. Chromosomal patterns of control oocytes following 1 h incubation and of those exposed to cryoprotectants, vitrified in conventional straws, and vitrified in open pulled straws (OPS) after 1 h incubation post-dilution

<table>
<thead>
<tr>
<th>Conditions</th>
<th>No. of oocytes</th>
<th>Chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>57</td>
<td>56 (98)</td>
</tr>
<tr>
<td>Exposed</td>
<td>63</td>
<td>60 (95)</td>
</tr>
<tr>
<td>Straws</td>
<td>67</td>
<td>52 (78)(^a)</td>
</tr>
<tr>
<td>OPS</td>
<td>55</td>
<td>48 (87)(^b)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

\(^a\) P < 0.05, compared with control oocytes and those exposed to cryoprotectants.

\(^b\) P < 0.05, compared with control oocytes.

Oocytes is not clear. In this study, it was found that mouse oocytes vitrified in OPS had a smaller percentage of morphological survival than those in conventional straws. It is considered that a portion of oocytes in OPS might come into contact directly with liquid nitrogen which may have had an adverse effect on their survival. In addition to type of tube or straw, the damage to oocytes during vitrification has been demonstrated to be dependent on the interaction of all the steps, including the rate of increase of exposure of the cells to the highly concentrated solutes, the temperature and time of exposure, and methods for diluting out the solutes (O’Neal et al., 1997). Further studies are needed to improve the survival rate of oocytes vitrified in small pulled straws.

When analysed immediately after dilution, oocytes exposed to cryoprotectants displayed dramatic alterations of their spindles. The ethylene glycol-based cryoprotectants could have been toxic to spindles and did not protect the spindles when the oocytes were dehydrated and rehydrated. After 1 h incubation, repolymerization of the microtubules partially occurred so that 84% of the oocytes recovered their normal spindles. Cooling could have further injured the microtubules in that oocytes vitrified in conventional straws had a smaller percentage of recovery of normal spindles. These findings were consistent with earlier observations in which the spindle organization of mouse oocytes partially recuperated after treatments with dimethyl sulphoxide-based cryoprotectants, slow freezing, or ultrarapid freezing (Aigner et al., 1992; Eroglu et al., 1998). In addition, in this study, oocytes vitrified in OPS had more normal spindles restored than did those in conventional straws. The former also apparently had more compact chromosomes than the latter at 1 h post-dilution. It is thought that appropriate organization of microtubules is essential for the alignment of chromosomes, and disorganization of the spindle may lead to chromosomal dispersion.

The tip of OPS is designed to have a small diameter and thin wall. Oocytes held in OPS with a very small volume of vitrification solution achieve a faster cooling and warming rate (a theoretical rate of 20 000°C/min) than those in conventional straws (2500°C/min) (Rall and Fahy, 1985; Vajta et al., 1998). They can rapidly traverse the temperature damaging to the spindle, assumed to be 15 to −15°C (Martino et al., 1996). Moreover, oocytes in vitrification medium (1-2 µl) in OPS are directly warmed in the dilution solution and are quickly expelled (within ~1 s) and immediately diluted. That reduces exposure to inappropriate temperatures and concentrated cryoprotectants. In contrast, the conventional straw is warmed in water and then cut with scissors. The oocytes in vitrification medium (65–70 µl) are expelled into the dilution solution and then placed into another dilution solution. It takes more time to pass through the unsuitable conditions (~45 s). These effects may explain why vitrification of oocytes using OPS preserves spindles better than that using conventional straws. It may also partly elucidate the finding of Vajta et al. (1998) that the developmental capacity of vitrified bovine oocytes could be improved using OPS in comparison with conventional straws. Martino et al. (1996) put bovine oocytes on electron microscope grids for vitrification, achieving a faster cooling rate (a theoretical rate of 180 000°C/min), and they also found a greater percentage of blastocyst formation than with conventional straws. The advantages and disadvantages of OPS and grids for the vitrification of oocytes deserve further studies.

The microtubular system of oocytes is crucial for fertilization and normal development (Pickering et al., 1990; Joly et al., 1992). The visual changes in the spindle after vitrification have been demonstrated to be linked to functional effects of oocytes on fertilization and development (O’Neil et al., 1997). Eroglu et al. (1998) found that insemination immediately after thawing of oocytes cryopreserved by a slow freezing method caused a delay in the dynamics of spindle rotation, second polar body formation, pronuclear formation, migration, and formation of the mitotic spindle. In contrast, insemination after 1 h incubation resulted in fertilization dynamics similar to those of controls. It was thought that a deliberate incubation period after thawing led to a sufficient recovery of microtubules and reduced the stress of cryopreservation prior to insemination. In our study, the oocytes vitrified either in conventional straws or OPS did partially recover the spindle system after post-dilution incubation for 1 h. The process of repolymerization of microtubules may be time-dependent (Aigner et al., 1992). Whether oocytes incubated longer can further recover the spindle and what the most adequate time for insemination is deserve further investigations.

This study investigated the effects of vitrification using conventional straws and OPS on microtubular organization and dynamics in mouse oocytes. Oocytes examined immediately post-dilution displayed profound spindle alterations that
may affect chromosomal alignment. Incubation for 1 h allowed recovery of normal spindle and chromosomes to various degrees. This study has demonstrated that vitrification of mouse oocytes using OPS preserves the spindle morphology and chromosomal pattern better than in conventional straws. Attaining more rapid cooling, warming, and dilution using OPS can alleviate the problem of injury to the spindle, which is very sensitive to cryoprotectants and low temperature. However, the morphological survival rate of oocytes using OPS is lower. The reason for this is not clear, but may be attributed to the direct contact of some oocytes with liquid nitrogen. Further research is warranted to improve the survival and preservation of the spindle in oocyte vitrification using small pulled straws. The OPS method results in possible exposure of the oocytes to liquid nitrogen, and a potential risk of infection. This may not be important for animal studies. For any clinical application of the method the aseptic condition and avoidance of contamination should be specially addressed.

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Spindle of oocytes after vitrification in OPS