Mouse embryos generated from frozen–thawed oocytes can successfully survive a second cryopreservation

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BACKGROUND: To determine whether mouse embryos generated from frozen–thawed oocytes can successfully survive a second cryopreservation. METHODS: Immature C57BL6*BALB/c female mice underwent superovulation and the collected oocytes were divided into three groups. Group A oocytes (n = 107) underwent IVF. Group B oocytes (n = 167) underwent IVF and embryos generated were then cryopreserved. Group C oocytes (n = 94) were cryopreserved, thawed and underwent IVF. Two–four-cell stage embryos were re-cryopreserved and thawed. Embryos from all groups were then cultured to the blastocyst stage. RESULTS: Cleavage rates to the 2–4-cell stage were 78, 71 and 46% for groups A, B and C respectively. Blastulation rates from 2–4 cell-stage embryos were 37/83 (45%), 27/118 (23%) and 8/35 (23%) for groups A, B and C respectively. Development to blastocysts was observed in 37/107 oocytes (35%), 27/167 oocytes (16%) and only 8/94 oocytes (9%) for groups A, B and C respectively. CONCLUSION: Oocyte cryopreservation results in reduced fertilization rates. Embryo cryopreservation reduces blastulation rates by half regardless of whether the oocytes were fertilized fresh or frozen–thawed. Nevertheless, embryos generated from cryopreserved oocytes can survive cryopreservation and develop to the blastocyst stage at rates comparable with embryos obtained from fresh oocytes.

Key words: blastocyst/cryopreservation/IVF/mouse/oocyte

Introduction

Gamete and embryo cryopreservation expand the possibilities of modern assisted reproductive techniques. It is well recognized that the ability to cryopreserve unfertilized human oocytes would make a significant contribution to infertility treatments. Oocyte cryopreservation is a possible solution for the ethical problems related to embryo storage, and the only available technique for preserving fertility in young women who have to undergo chemotherapy or radiotherapy. Pregnancies from cryopreserved oocytes have been reported after thawing, insemination and transfer of the subsequent embryos (Porcu et al., 2000). The recently reported pregnancies obtained by human oocyte cryopreservation are encouraging (Wininger and Kort, 2002).

Oocyte cryopreservation has been applied with varying success to a number of different species including the human (Gook et al., 1993; Bouquet et al., 1995). Several studies typically reported different rates of survival (20–80%), fertilization (30–60%) and cleavage (32–100%) in human oocytes (Fabbri et al., 2000). This variability of results raises doubts regarding the usefulness of oocyte cryopreservation in IVF treatment cycles. The main problems with oocyte cryopreservation concern the survival and fertilization rates although the introduction of ICSI led to an increase in these rates. Further investigation of various biophysical changes during oocyte cryopreservation could improve success rates.

Recently, fundamental studies on the effects of cooling, membrane permeability, cryoprotectant addition and ice formation have been performed on human oocytes (Wininger and Kort, 2002). It is likely that successful human oocyte cryopreservation will only follow once these factors are fully understood, but the existing base of knowledge should provide a platform for further improvements in the techniques currently employed. The accurate determination of the freezing conditions that promote intracellular ice formation is crucial for designing cryopreservation protocols for oocytes (Trad et al., 1999).

If better oocyte survival after cryopreservation is obtained, an increased fertilization rate would be anticipated with an increased number of embryos being found suitable for transfer. All spare embryos generated from the frozen–thawed oocytes could then be subjected to cryopreservation followed by thawing when needed. Thus, embryos generated from these frozen oocytes would undergo freezing–thawing more than once. The aim of this study was to assess the efficacy of this process in a mouse model.
Materials and methods

Superovulation and study groups

Approval from the animal facility ethics committee at the Hebrew University Medical School was obtained. CB6F1 (C57BL6*BALB/c) female mice aged 4–5 weeks were injected i.p. with 5 IU of pregnant mare’s serum gonadotrophins (PMSG) (Sigma Chemical Co., USA) followed by 5 IU of hCG (Chorigon; Teva, Israel) given 46–48 h later. Cumulus–oocyte complexes (COC) were collected from the Fallopian tubes 12–14 h after hCG injection and transferred into M-2 medium (Sigma, USA) supplemented with 4 mg/ml bovine serum albumin (BSA) (Sigma, USA). The oocytes were randomly divided into the following three groups. Group A (control oocyte group): oocytes (n = 107) were inseminated and cultured to the blastocyst stage in Whittingham’s medium according to Hogan et al. (1994). Group B (embryo cryopreservation group): fresh oocytes (n = 167) underwent IVF and the resulting embryos were cryopreserved at the 2–4-cell stage, then thawed and cultured in Whittingham’s medium to the blastocyst stage (Whittingham, 1971). Group C (double-freezing group): oocytes (n = 94) were cryopreserved and thawed. Surviving oocytes underwent IVF and the developed embryos were frozen at the 2–4-cell stage. Subsequently, these embryos were thawed and assessed for their blastulation potential in Whittingham’s medium.

Oocyte cryopreservation

Oocytes in study group C were cryopreserved using a slow freezing method (Fab bri et al., 2001). Briefly, oocytes were denuded from corona and cumulus cells in M-2 solution (Sigma, USA) containing 4 mg/ml BSA and 300 µg/ml hyaluronic acid. Following denudation, oocytes were rinsed with PBS solution supplemented with 12 mg/ml human serum albumin (HSA) (Sigma, USA). Oocytes were then transferred to equilibration solution [PBS with 1.5 mol/l 1,2-propanediol (PROH) and 12 mg/ml HSA] for 10 min at room temperature, followed by 15 min in loading solution (PBS with 1.5 mol/l 1,2-PROH, 0.3 mol/l sucrose and 12 mg/ml HSA) at room temperature. The oocytes were loaded into cryo-tubes containing 0.25 ml loading solution. Cryopreservation was performed using a slow-freezing protocol in a programmed biological freezer (Planer Kryo 10; Planer products Ltd, UK) using the following cryopreservation protocol: starting thawing temperature was 23°C, and the temperature was reduced at a rate of 2°C/min down to −7°C. At that point, manual seeding was performed. After seeding, the temperature was reduced by 0.3°C/min to −30°C followed by a decline rate of 50°C/min to −150°C. The tubes were then lowered to liquid nitrogen containers.

Thawing was performed by holding tubes in the air for 60 s and then in a 35°C water-bath for a further 90 s. Oocytes were moved into PBS with 1 mol/l PROH, 0.3 mol/l sucrose and 12 mg/ml HSA for 5 min at room temperature. The oocytes were transferred to PBS containing 0.5 mol/l PROH, 0.3 mol/l sucrose and 12 mg/ml HSA, for another 5 min at room temperature, followed by 10 min in PBS containing 0.3 mol/l sucrose and 12 mg/ml HSA. The oocytes were then transferred into PBS solution containing 12 mg/ml HSA for 10 min at room temperature and another 10 min in 37°C. Finally, the oocytes were transferred into Whittingham’s medium for incubation at 37°C at 5% CO₂ in air.

Oocyte survival was determined by the presence of an intact zona pellucida and a healthy-looking cytoplasm. Survival was also determined by re-expansion of the oocyte leaving a small normalized perivitelline space.

IVF

Epididyma were collected from 10–12 week old CB6F1 males, and sperm were recovered into Whittingham’s medium by pressing each cauda epididymis with a pair of forceps. The sperm were incubated (37°C, 5% CO₂ in air) in Whittingham’s medium under paraffin oil for 1.5 h to allow capacitation.

Control as well as frozen–thawed oocytes were transferred to plates containing 200 µl Whittingham’s medium (10–12 oocytes in each drop), covered by light paraffin oil, and incubated until insemination. Sperm were added to the insemination plates at a final concentration of 1–2×10⁶ cells/ml. For groups A and B, insemination was performed 13.5–15 h after hCG injection. In the double-freezing group (group C), only oocytes which survived the cryopreservation were inseminated.

Fertilization and embryo growth

Four hours after insemination, oocytes were washed three times and transferred to 60 µl drops (10–12 oocytes in each drop) of Whittingham’s medium covered with paraffin oil. On the following day, the number of oocytes that had reached the 2-cell stage was recorded. These 2–4-cell embryos were further cultured for 72 h, and the rate of blastocyst formation was determined. Assessment of cleavage and embryo development was performed employing inverted contrast microscopy.

Embryo cryopreservation

Embryos from groups B and C were cryopreserved using a slow-freezing technique. The embryos were placed in HTF–HEPES medium containing 12 mg/ml HSA (enriched HTF-Hepes) with 1.5 mol/l PROH for 10 min at room temperature. They were then transferred into cyro-tubes containing 0.25ml of enriched HTF-HEPES medium containing 1.5 mol/l PROH and 0.1 mol/l sucrose, and placed in the programmable freezer. The freezing protocol was the same as for oocytes. Thawing was performed for 60 s in air, and then in a water-bath at 35°C for 90 s. The embryos were transferred to a series of solutions (5–10 min each) containing 0.2 mol/l sucrose and reducing concentrations of PROH (1.0, 0.5, 0 mol/l). The embryos were washed in M-2 medium with 4 mg/ml BSA at room temperature, followed by 10 min incubation in this medium at 37°C. The embryos were finally cultured in Whittingham’s medium.

Blastocyst culture

Fresh (group A) as well as frozen–thawed (groups B and C) embryos were cultured in Whittingham’s medium, at 37°C, 5% CO₂ in air, and were allowed to develop to the blastocyst stage for an additional 72 h. The embryos were examined for morphology and cleavage rate. Examination was performed by the same investigator using Normarski’s inverted optics. Good morphology blastocysts were classified as those with a distinct inner cell mass (ICM), a well-differentiated trophoderm, and a single large blastocoelic cavity on day 3 of development.

Statistical analysis

Statistical analysis was performed using Pearson’s χ²-test. Blastulation rates were compared also with the statistical linear-by-linear test. P < 0.05 was considered as statistically significant.

Results

Cleavage rates for groups A, B and C were 83/107 (78%), 118/167 (71%) and 35/77 (46%) respectively. Group C originally included 94 oocytes of which 77 (82%) survived the slow-freezing procedure and were used for the study. Cleavage rate of cryopreserved oocytes (46%) was significantly (P < 0.01) lower when compared to that obtained in fresh oocytes of

Frozen-thawed mouse oocytes can be re-frozen
groups A or B. The cleavage rates in groups A and B were comparable at 78 and 71% respectively (Table I).

Development rates of oocytes to the blastocyst stage were 37/107 in group A (35%), 27/167 in group B (16%) and only 8/94 (9%) in group C. These rates differ significantly between group A and group B or C (P < 0.01) (Table I). These results show linear association (P < 0.001), demonstrating that each cryopreservation step added to the procedure significantly lowered blastulation rate.

Blastulation rate in group A was seen in 37/83 2–4-cell embryos (45%). This was significantly different (P < 0.01) from groups B and C where development to the blastocyst stage from 2–4-cell stage embryos was 27/118 (23%) in group B and similarly 8/35 (23%) in group C (Table I). Thus frozen–thawed embryos attained similar blastulation rates regardless of whether they originated from fresh (group B) or frozen–thawed (group C) oocytes.

Discussion

Various attempts to cryopreserve human oocytes have been performed with conflicting results. Thus, although human oocyte cryopreservation has been attempted for more than two decades (Chen, 1986), this technique is still considered an experimental one.

In the present study we demonstrate that cryopreservation of mouse oocytes results in a 32% decrease in cleavage rate (from 78 to 46%). This finding has already been reported (Mandelbaum, 1991; Wood et al., 1992; Gook et al., 1993; Baka et al., 1995; Bouquet et al., 1995; Eppig and O'Brien, 1996) and reflects damage to the oocyte’s intracellular organelles, plasma membrane and the zona pellucida.

Nevertheless, it appears that tolerance for freezing and thawing differs significantly between human and mouse oocytes. Opposing results, in favour of human oocytes, were found when compared with mouse oocytes cryopreserved by the same method (Gook et al., 1993). Human oocytes are thus also expected to withstand double cryopreservation better.

The meiotic spindle of mature oocytes has been reported to suffer significant damage during cryopreservation (Pickering and Johnson, 1987; Baka et al., 1995). However, fluorescence microscopy has demonstrated that most oocytes that survived cryopreservation had a normal spindle (Gook et al., 1993) and normal chromosomal status (Gook et al., 1994). Other targets of oocyte cooling damage include damage to cortical granule vesicles (Vincent and Johnson, 1992), aneuploidy (Bouquet et al., 1995), zona hardening (George and Johnson, 1993) and chilling damage which is associated with Ca2+ oscillation (Bend-Yosef et al., 1995).

Nevertheless, recent advances (Fabbri et al., 2001; Winerger and Kort, 2002) indicate that the clinical applications of oocyte cryopreservation could broaden in the future. This will result in a larger number of embryos obtained from frozen–thawed oocytes. Since the worldwide tendency is to reduce the number of embryos transferred to the uterus to minimize multiple pregnancies, the demand for embryo cryopreservation is on the rise (Mandelbaum et al., 1998). The need to cryopreserve embryos obtained from frozen–thawed oocytes prompts the debate on the effects of double freezing. Little is known about these effects. Cryopreservation of mouse embryos was shown to increase the rates of fertilization failure (Carroll et al., 1989) and polyplody (Glenister et al., 1987; Bouquet et al., 1995).

No study has previously prospectively examined the outcome of double oocyte freezing. A few human clinical pregnancies following two embryo freeze–thaw cycles have been reported (Check et al., 2001; Farhat et al., 2001; Yokota et al., 2001; Estes et al., 2003). Normal in vitro development from 8–16-cell mouse embryos to the blastocyst stage was reported even after three successive embryo freeze–thaw cycles (Vitale et al., 1997). Our study is the first to compare double freezing of oocytes with embryo cryopreservation results. The endpoint chosen was development to the blastocyst stage and thus reflects potential late effects on the oocytes. Moreover, development to the blastocyst stage is known to show a high correlation with the chance of attaining pregnancy. Future experiments will be important to test the pregnancy rates after embryo transfer using such embryos.

The question we tried to answer was whether the oocyte damage caused by the cryopreservation process would present beyond the fertilization. We hypothesized that if the oocyte suffered severe damage in the freezing process, it would have a significantly smaller chance of reaching the blastocyst stage compared with non-frozen oocytes. However, our results have clearly shown that although cryopreserved oocytes had only a 9% chance of attaining the blastocyst stage, this was due to a decrease in fertilization. Indeed the oocytes that successfully fertilized and were re-frozen reached blastulation rates similar to those of fresh oocytes which were frozen–thawed at the 2-cell stage. Taken together, these results clearly show that damage to the oocyte organelles and membranes only prevents fertilization. If fertilization takes place, frozen–thawed oocytes behave like fresh ones.

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### Table I. Cleavage rates and development to the blastocyst stage in mouse embryos obtained from three different groups; embryos developed from fresh oocytes (group A), embryos frozen at the 2–4-cell stage (group B) and embryos obtained from frozen–thawed oocytes, which then underwent a second freezing (group C)

<table>
<thead>
<tr>
<th>Groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of retrieved oocytes</td>
<td>107</td>
<td>167</td>
<td>94</td>
</tr>
<tr>
<td>No. of oocytes lost in cryopreservation–thawing process</td>
<td>–</td>
<td>–</td>
<td>17</td>
</tr>
<tr>
<td>% from total oocytes</td>
<td>18</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>No. of 2–4-cell embryos</td>
<td>83</td>
<td>118</td>
<td>35</td>
</tr>
<tr>
<td>% from intact oocytes</td>
<td>78</td>
<td>71</td>
<td>46</td>
</tr>
<tr>
<td>No. of blastocysts obtained</td>
<td>37</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td>% from total oocytes</td>
<td>35</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>% from cleaved embryos</td>
<td>45</td>
<td>23</td>
<td>23</td>
</tr>
</tbody>
</table>

a,b,cDiffer significantly between group C and groups A or B.

a,b,cDiffer significantly between group A and groups B or C.
References


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