Germinal vesicle transfer between fresh and cryopreserved immature mouse oocytes

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BACKGROUND: We assessed the maturational competence and the chromosomal pattern of mouse oocytes reconstructed by germinal vesicle (GV) transfer technique using nuclear and/or cytoplasmic components from cryopreserved GV stage oocytes. METHODS: From 657 GV oocytes (326 fresh and 331 frozen/thawed), four groups of reconstructed oocytes were obtained by micromanipulation and electrofusion: fresh GV–fresh cytoplast (FF), thawed GV–thawed cytoplast (TT), fresh GV–thawed cytoplast (FT), thawed GV–fresh cytoplast (TF). All reconstructed oocytes were cultured in vitro to metaphase II. RESULTS: Survival rate after manipulation and electrofusion, as well as progression to metaphase II, did not differ significantly among the four groups. Comparing reconstructed oocytes with fresh and thawed control pools, the only difference was a slightly but significantly higher maturation rate in the TT pool versus matched controls (P < 0.01). Cytogenetic analysis of 25 reconstructed oocytes showed the expected number of 20 chromosomes in 88% of them. CONCLUSIONS: We conclude that both nuclear and cytoplasmic components derived from cryopreserved immature oocytes are suitable for GV transfer procedure, and generate chromosomally normal oocytes able to progress to metaphase II in vitro. The possibility of using cryostored immature oocytes as a source of nuclei and cytoplasm could help in applying GV transfer procedure, both in research and clinical settings.

Key words: cryopreservation/germinal vesicle transfer/in-vitro maturation/nuclear transfer/oocyte

Introduction

Germinal vesicle (GV) transfer technique is a useful tool to study the interaction between nucleus and cytoplasm in the maturation process of mammalian oocytes (Fulka et al., 1996; Liu et al., 1999; Takeuchi et al., 1999; Zhang et al., 1999). Using micromanipulation and electrofusion procedures it is possible to reconstruct an oocyte by transferring a GV (karyoplast) into a cytoplasm (cytoplast) derived from an enucleated donor oocyte at the same developmental stage (Liu et al., 1999; Zhang et al., 1999; Takeuchi et al., 2001). Mouse oocytes reconstructed by sequential transfer of GV and haploid pronuclei have been shown to develop to the blastocyst stage in vitro and can result in normal live offspring when transferred to the oviducts of foster mothers (Liu et al., 2000a,b).

As a clinical or research procedure, GV transfer presents a major logistical problem due to the difficulty of obtaining donor and recipient oocytes at the same GV stage. If frozen oocytes could be used, the proper combination of karyoplast and cytoplasm from different sources would be easier. While long-term cryopreservation of pre-implantation embryos is a well-established technique in many mammalian species, the same survival rate has not yet been achieved with frozen/thawed oocytes (Paynter, 2000). Although normal offspring were recently obtained through the fertilization of frozen/thawed MII human oocytes (Porcu et al., 2000), there are few detailed studies on the cryopreservation of immature, GV stage oocytes (Cha et al., 2000; Fabbri et al., 2000; Goud et al., 2000).

In the present study conducted with a mouse model, we evaluated the in-vitro maturation (IVM) of GV stage oocytes reconstructed by GV transfer in the following conditions: (i) fusion of a fresh GV and a fresh cytoplast, (ii) fusion of a frozen/thawed GV and a fresh cytoplast, (iii) fusion of a fresh GV and a frozen/thawed cytoplast, and (iv) fusion of a frozen/thawed GV and a frozen/thawed cytoplast. The chromosomes of some reconstructed oocytes were also examined to assess whether cryopreservation and/or GV transfer techniques could allow a normal in-vitro meiotic maturation.

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Materials and methods

Collection of mouse GV oocytes
Female CD-1 mice 6–8 weeks old were superovulated by a single i.p. injection of 5 IU of pregnant mare serum gonadotrophin (PMSG; Sigma, St Louis, MO, USA). Between 36 and 48 h post-injection the animals were killed by neck dislocation, their ovaries were removed and GV oocytes were collected by puncturing the follicles in a 60 mm diameter culture dish (Falcon, Oxnard, MA, USA) containing 1 ml of modified human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT, USA). Cumulus cells were mechanically removed from oocytes by gentle, repeated pipetting. The harvested oocytes were then divided into three groups and utilized either for cryopreservation, micromanipulation or IVM.

IVM of GV oocytes
In order to find the most appropriate culture medium for IVM of mouse oocytes, we compared two different culture media. Two pools of fresh GV stage oocytes were cultured at 37°C in 5% CO₂ for 16–20 h; the first pool (157 oocytes) was incubated in HTF plus 10% FCS, the other pool (155 oocytes) in HTF plus 10% FCS supplemented with 35 ng/ml insulin (Sigma) and 0.07 IU/ml Pergonal® (FSH:LH 1:1 v:v; Serono, Switzerland). The morphological appearance of the first polar body extrusion after 16–20 h incubation was the criterion used to assess nuclear maturation of GV oocytes. Since there was no difference in the oocyte maturation rate between the two culture media, all the following experiments utilized GV oocytes cultured in HTF + 10% FCS without insulin and/or gonadotrophins.

Freezing, cryopreservation and thawing of GV oocytes
A slow-freeze/rapid-thaw procedure was used to cryopreserve the GV oocytes (Lassalle et al., 1985). Phosphate buffered saline (PBS; SAGE Biopharma, Bedminster, NJ, USA) supplemented with 10% FCS, 1.5 mol/l 1,2-propanediol (PROH; Sigma) and 0.1 mol/l sucrose (Sigma) was used. Before freezing, the oocytes were mechanically denuded with a Pasteur pipette and progressively equilibrated in solution A (PBS + 10% FCS) for 5 min at 37°C, then in solution B (solution A supplemented with 1.5 mol/l PROH) for 15 min at 23°C, and finally in solution C (solution B supplemented with 0.1 mol/l sucrose) for 5 min at 23°C. After equilibration, 20–30 oocytes were placed in a 1.5 ml sterile plastic vial (Nunc A/S, Roskilde, Denmark), containing 0.5 ml of solution C. The vials were loaded into a programmable, controlled rate-freezing machine (Planer Kryo 10 Series II; T.S. Scientific, Perkasie, PA, USA) and the temperature was reduced from 23°C to −7°C at a cooling rate of −2°C/min. Ice nucleation was induced manually by seeding each vial with pre-cooled forceps. After seeding, the vials were kept for 5 min at the seeding temperature and then cooled at a rate of 0.3°C/min until reaching −32°C. Each vial was then plunged directly into liquid nitrogen and stored in a tank for 4–30 days before thawing.

Thawing was performed by direct immersion of the frozen cryovials into 31°C water bath. The cryoprotectants were removed stepwise by washing the oocytes for 5 min in solutions containing a decreasing PROH concentration (1.5 mol/l, 1 mol/l, 0.5 mol/l), with a final one-step dilution of the sucrose in PBS + 10% FCS.

After thawing, oocytes were divided into two pools. The first (thawed controls; TC) was immediately transferred in culture dishes containing 1 ml HTF + 10% FCS and cultured as previously described, the second pool was submitted to micromanipulation.

Micromanipulation: preparation of karyoplasts and cytoplasm for GV transfer
At 30 min before micromanipulation, GV oocytes were incubated at room temperature in microdrops of modified HTF plus 10% FCS, supplemented with 3-isobutyl-1-methylxanthine (IBMX) (50 µg/ml; Sigma) and 7.5 µg/ml cytochalasin B (Sigma). This IBMX concentration prevents the germinal vesicle breakdown (GVBD) without affecting oocyte viability and progression to metaphase II (Liu et al., 1999). Cytochalasin B was used to increase the oolemmal elasticity prior to enucleation.

Utilizing a Narishige micromanipulator mounted on an inverted microscope (Nikon Eclipse TE300®; Narishige, Melville, NY, USA), the zona pellucida was lanced with a sharp-tipped pipette and the GV was gently aspirated into a bevelled glass pipette with an inner diameter of 20 µm (Figure 1A, B). Each GV was surrounded by a small amount of cytoplasm (karyoplast), and appeared to be encapsulated by a membrane. Cytoplasts (cytoplasms of enucleated cells) were obtained by enucleating GV stage oocytes with the same procedure. Karyoplasts were transferred individually into the perivitelline space of the previously prepared cytoplasts by micro-injection (Figure 1C, D), and the obtained GV-cytoplast complexes (Figure 2) were incubated for 15 min in HTF medium with 10% FCS (37°C; 5% CO₂) prior to electrofusion.

Electrofusion of GV–cytoplast complexes
The GV–cytoplast complexes were placed in the fusion medium (0.3 mol/l mannitol, 0.1 mmol/l CaCl₂, 0.05 mmol/l MgSO₄ and 0.05 mg/ml BSA in deionized water) between the platinum electrodes of a fusion chamber. They were first aligned with an AC pulse of 11 volts for 7–10 s, and then fused with a DC electrical pulse of 1.8–2 kVols/cm for 50 µs. The fusion procedure was repeated three times with an interval of 30 min between pulses and was performed using a Model 2001 Electro Cell Manipulator® (BTX Inc., San Diego, CA, USA). The amplitude of electropulses given to the chamber was analysed by a Model 500 Graphic Pulse Analyzer® (BTX). The incorporation of GV nuclei into the cytoplasm was monitored by time-lapse microscopy.

Experimental design
Micromanipulation and electrofusion were used to create the following four pools of reconstructed oocytes: fresh GV–fresh cytoplast (FF), thawed GV–thawed cytoplast (TT), fresh GV–thawed cytoplast (FT), thawed GV–fresh cytoplast (TF). After electrofusion, the reconstructed oocytes were washed twice in modified HTF medium and then incubated in HTF with 10% FCS at 37°C, 5% CO₂.

The reconstructed oocytes were matured in vitro as previously described (Liu et al., 1999). Progression to MII was assessed by extrusion of the first polar body after 16–20 h of incubation. Pools of non-manipulated, fresh GV oocytes [fresh controls (FC); n = 218] and non-manipulated, frozen/thawed GV oocytes [thawed controls (TC); n = 275] were incubated in the same conditions and used as controls. Some frozen/thawed GV oocytes underwent electrostimulation with the same protocol described above, and were used as controls to assess the effect of the electrostimulation per se on the oocyte nuclear maturation (TCE controls, n = 42).

Cytogenetic analysis
In order to assess whether meiosis progressed normally from GV to MII stage, 25 FT and TF reconstructed oocytes that extruded the first polar body were submitted to cytogenetic analysis according to Tarkowski (Tarkowski, 1966). The oocytes were first placed into a 1% sodium citrate hypotonic solution for 10 min at 23°C and then fixed on a grease-free slide with three different solutions of methanol, acetic acid and water. The spread chromosomes were stained with Hoechst 33342 (5 µg/ml in PBS). Chromosome structure and number were determined by fluorescence microscopy (Figure 4).
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Figure 1. GV transfer technique: lancing of the zona pellucida (A), removal of the karyoplast (germinal vesicle, GV) from the oocyte (B), and transfer of the karyoplast into the perivitelline space of a previously prepared cytoplast by micro-injection (C and D).

Figure 2. Cytoplast–karyoplast complexes obtained from fresh and/or frozen GV stage oocytes: the karyoplast is in the perivitelline space of the recipient enucleated oocyte, prior to electrofusion.

Statistical analysis
Data were analysed using the \( \chi^2 \) test with significance accepted at \( P < 0.05 \).

Results
The overall oocyte survival rate for the freeze/thaw procedure was 76% (331/436). A total of 657 GV oocytes (326 fresh and 331 frozen/thawed) were included in the study and underwent micromanipulation. Overall, 82% survived manipulation, resulting in 540 GV–cytoplast complexes that were divided into four different pools: fresh GV–fresh cytoplast (FF; \( n = 135 \)), thawed GV–thawed cytoplast (TT; \( n = 138 \)), fresh GV–thawed cytoplast (FT; \( n = 143 \)), thawed GV–fresh cytoplast (TF; \( n = 124 \)). No significant difference in the survival rate after manipulation was observed among these four pools of oocytes (range 79–85%; Table I). The fusion rate of the GV–cytoplast complexes also showed no significant difference among the four pools (range 40–51%; Table I). Thus, the resulting reconstitution efficiency (number of successfully fused oocytes out of the number of manipulated oocytes) was not statistically different among the four study groups: FF 32% (55/170), TT 35% (57/162), FT 43% (73/169), 31% (49/156).

The maturation rate of each pool of reconstructed oocytes was compared with that of non-manipulated, fresh and thawed control pools (Table II). The maturation rates of pools FF, FT and TF (80, 86, and 78% respectively) did not differ significantly from those of the control pools (77% for CF and 78% for CT respectively). However, the TT oocyte pool maturation rate was significantly higher with respect to control CT (91 versus 78%; \( P < 0.01 \)). This finding was not due to electrostimulation per se, as the maturation rate in the TT pool did not differ significantly from that observed in non-manipulated, thawed, electro-stimulated controls (TCE, \( n = 42 \); Table II). GV breakdown takes place after 1–3 h of in-vitro culture, and both the reconstructed and control oocytes reach the MII stage after 16–20 h of incubation (Liu et al., 1999).

DNA staining with Hoechst 33342 routinely uncovered two fluorescent areas, one in the cytoplasm and one in the polar
Figure 3. Electrofusion of the karyoplast and the cytoplast: progressive incorporation of the karyoplast into the cytoplast after electrofusion (time from 1 to 4: approximately 30 min).

Figure 4. Chromosome spread of a normal MII oocyte matured in vitro from a reconstructed GV stage oocyte (chosen from group FT = fresh GV–thawed cytoplast): staining with Hoechst 33342 shows 20 univalent chromosomes (in the centre) and dispersed polar body chromosomes (bottom right quadrant).

body. Cytogenetic analysis showed the expected number of 20 chromosomes in both the nucleus and the polar body in 88% (22/25) of the oocytes tested, indicating that oocytes reconstructed with frozen–thawed nucleus and/or cytoplasm mature normally in vitro to the MII stage.

Discussion

It was previously shown that it is possible to reconstruct a normal GV oocyte by using micromanipulation to remove the GV from a fresh oocyte and electrofusion to transfer it into
and fusion rates of frozen/thawed oocytes were comparable of these oocytes, since both the post-manipulation survival freezing/thawing process did not damage the cell membrane also appears to be adequate for cryopreserving immature mouse 1,2-propanediol as a cryoprotectant and includes slow cooling, human zygotes. This freezing/thawing procedure, which uses Lassalle and colleagues (Lassalle et al. to support nuclear maturation.

Our freezing/thawing protocol was adapted from that of Lassalle and colleagues (Lassalle et al., 1985), which was originally developed for the cryopreservation of mouse and human zygotes. This freezing/thawing procedure, which uses 1,2-propanediol as a cryoprotectant and includes slow cooling, also appears to be adequate for cryopreserving immature mouse oocytes, since we observed a high survival rate. Moreover, the freezing/thawing process did not damage the cell membrane of these oocytes, since both the post-manipulation survival and fusion rates of frozen/thawed oocytes were comparable with those of fresh ones. The electrofusion procedure appears to be the primary factor that determines reconstitution efficiency, but it is important to observe that this is not depending on cryopreservation per se as the electrofusion rates did not differ among the four groups of reconstructed oocytes. In previous studies the reconstitution rate ranged from 20% (Zhang et al., 1999), to 73% (Liu et al., 1999), to 87% (Takeuchi et al., 1999). Such differences among authors and in the present study (range 31–43%) are probably due to different fusion media compositions as well as different voltage and length of electropulses applied.

The maturation rate of reconstructed GV oocytes was unaffected by freezing, as no significant differences in the maturation rate were observed comparing oocytes reconstructed using fresh or thawed cytoplasm and/or karyoplast. Rather surprisingly, GV oocytes reconstructed with thawed cytoplasm and thawed karyoplast achieved the best maturation rate, a rate that was also significantly higher than that observed with non-reconstructed, thawed control oocytes. A possible explanation for this finding is that the sequence of cryopreservation, manipulation and fusion procedures may result in the selection of the ‘fittest’ oocytes which are more likely to achieve nuclear maturation in vitro.

Cytogenetic analysis showed that the use of a cryopreserved cytoplasm and/or karyoplast does not affect the ability of the reconstructed GV oocytes to reach a normal MII stage. Thus, the ability of GV ooplasm to support a normal first meiotic division is not compromised by the cryopreservation procedures. However, the ability of GV oocytes reconstructed from cryopreserved karyoplasts or cytoplasm to support subsequent embryonic development to term remains to be determined. However, live births have been recently reported for following GV transfers using fresh oocytes (Li et al., 2001).

GV transfer between fresh and frozen/thawed oocytes, either using cryopreserved karyoplast or donor’s cytoplasm, might be an option to preserve fertility in young cancer patients or even in young women with a family history of premature ovarian failure. In these subjects, fragments of ovarian tissue could be retrieved and cryopreserved before chemo- or radiotherapy to be used subsequently as a source of GV from primordial follicles. While freezing human ovarian tissue is comparatively easy (Hovatta et al., 1996; Oktay et al., 1997), it is technically difficult to achieve complete in-vitro maturation of human follicles from the primordial stage up to ova- lation (Smits and Cortvriendt, 1999; Wright et al., 1999). Our results suggest that this cumbersome in-vitro step may be eventually bypassed by transferring immature nuclei from frozen/thawed primordial follicles directly into cytoplasts obtained from donor fresh or thawed oocytes. At present, this is only a hypothesis. In fact, although the possibility of cryopreserving human ovarian fragments with an acceptable survival rate of primordial follicles has been previously demonstrated (Hovatta et al., 1996; Oktay et al., 1997), the GV of a primordial follicle is probably not equivalent to the GV of a pre-ovulatory oocyte because maternal imprinting and its allele specific modifications in DNA methylation are established during the intervening period of oocyte growth (Kono et al., 1996; Bao et al., 2000). Recently, the application of ooplasmic injection has been

### Table I. Survival and fusion rate after manipulation of fresh and/or thawed oocytes

<table>
<thead>
<tr>
<th>Type of GV–cytoplast complex</th>
<th>No. manipulated</th>
<th>Survival after manipulation (%)</th>
<th>GV–cytoplast complex fused (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group FF</td>
<td>170</td>
<td>135 (79)</td>
<td>55 (41)</td>
</tr>
<tr>
<td>Group TT</td>
<td>162</td>
<td>138 (85)</td>
<td>57 (41)</td>
</tr>
<tr>
<td>Group FT</td>
<td>169</td>
<td>143 (85)</td>
<td>73 (51)</td>
</tr>
<tr>
<td>Group TF</td>
<td>156</td>
<td>124 (79)</td>
<td>49 (40)</td>
</tr>
</tbody>
</table>

**FF** = fresh GV–frozen cytoplast; **TT** = thawed GV–thawed cytoplast; **FT** = fresh GV–thawed cytoplast; **TF** = thawed GV–frozen cytoplast.

### Table II. Progression to metaphase II of reconstructed fresh and/or cryopreserved oocytes and controls

<table>
<thead>
<tr>
<th>Type of GV–complex</th>
<th>No. reconstructed</th>
<th>Progression to MI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group FF</td>
<td>55</td>
<td>44 (80)</td>
</tr>
<tr>
<td>Group TT</td>
<td>57</td>
<td>52 (91)*</td>
</tr>
<tr>
<td>Group FT</td>
<td>73</td>
<td>63 (86)</td>
</tr>
<tr>
<td>Group TF</td>
<td>49</td>
<td>38 (78)</td>
</tr>
<tr>
<td>Group CF</td>
<td>218</td>
<td>168 (77)</td>
</tr>
<tr>
<td>Group CT</td>
<td>275</td>
<td>215 (78)</td>
</tr>
<tr>
<td>Group CTE</td>
<td>42</td>
<td>36 (86)</td>
</tr>
</tbody>
</table>

**FF** = fresh GV–frozen cytoplast; **TT** = thawed GV–thawed cytoplast; **FT** = fresh GV–thawed cytoplast; **CF** = control fresh; **CT** = control thawed; **CTE** = control thawed electrostimulated.

*Value is statistically different from CT (P < 0.01).
reported to rescue developmentally compromised oocytes in humans, but this procedure has also been found to generate mitochondrial DNA (mtDNA) heteroplasmy in some offspring (Barrit et al., 1999; Brenner et al., 2000). Despite the apparently normal health of these babies, there is little known about the long term consequences of such heteroplasmy. Although the presence or number of mitochondria in a GV karyoplast is not known, the possibility exists that two different mtDNA populations derived from the cytoplasm and karyoplast will result in the reconstructed oocyte and impact on their interactions with nuclear DNA. Future studies designed to investigate these issues are needed both for scientific and ethical reasons.

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