Simple and Efficient Vitrification Procedure for Cryopreservation of Mouse Embryos

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Abstract: Mouse pronuclear oocytes and 2-cell embryos derived from in vitro fertilization were cryopreserved by a novel simple vitrification procedure. Most cryopreserved oocytes/embryos were morphologically normal after warming, and 89–92% of them developed to the blastocyst stage during the culture. Moreover, the rate of morphologically normal pronuclear oocytes after being repeatedly cooled and warmed three times was as high as that of oocytes cooled and warmed only once, and 85% of them developed to the blastocyst stage. In addition, 43–57% of the cryopreserved oocytes/embryos transferred to recipients had developed normally to live fetuses observed on day 18.5 of pregnancy.

Key words: cryopreservation, mouse embryo, simple vitrification

Since the report by Whittingham et al. [10], the slow freezing and slow thawing method has been widely used for the routine cryopreservation of preimplantation embryos of many mammalian species. However, this method requires an expensive programmable freezing machine to achieve long periods of slow cooling, in addition to involving complicated and quite time-consuming procedures.

Successful cryopreservation of mouse oocytes/embryos at −196°C by an ultrarapid freezing procedure and by a simple vitrification method, a modified original vitrification method [5], has recently been reported [1–4, 6, 7]. The ultrarapid freezing method developed by Nakagata [2–4] is very simple, is not time-consuming, and reduces equipment costs. However, the handling of oocytes/embryos is difficult during the freezing and thawing because the oocytes/embryos have to be plunged directly into liquid nitrogen within 5–10 sec after the exposure to the cryopreservation solution at room temperature. At the recovery step, the cryopreservation solution must be diluted immediately after thawing to avoid the toxicity of the cryoprotectant on the oocytes/embryos.

By modifying the ultrarapid freezing method we have developed a simple and efficient vitrification method that only requires a brief two-step exposure of oocytes/embryos to the cryopreservation solution prior to plunging them directly into liquid nitrogen. Herein we report the viability of pronuclear oocytes and 2-cell embryos after cryopreservation by this simple vitrification procedure.

Animals: C57BL/6J, BDF1 (C57BL/6N × DBA/2N) and MCH:ICR mice were purchased from CLEA JAPAN Inc., Japan. C57BL/6J and BDF1 were used as oocytes for in vitro fertilization and MCH:ICR mice were used as pseudopregnant recipients for embryo

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Table 1. Survival rates of cryopreserved pronuclear oocytes and 2-cell embryos after warming

<table>
<thead>
<tr>
<th>Stage</th>
<th>Strain</th>
<th>Frozen*</th>
<th>Recovered* (n, %)</th>
<th>Morphologically normal* (n, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pronuclear oocyte</td>
<td>C57BL/6J</td>
<td>120</td>
<td>118 (98.3)</td>
<td>115 (97.5)</td>
</tr>
<tr>
<td>2-cell</td>
<td>C57BL/6J</td>
<td>317</td>
<td>317 (100)</td>
<td>315 (99.4)</td>
</tr>
<tr>
<td>Pronuclear oocyte*</td>
<td>BDF1</td>
<td>150</td>
<td>141 (94.0)</td>
<td>140 (99.3)</td>
</tr>
</tbody>
</table>

* Pronuclear oocytes were repeatedly cooled and warmed three times.

transfer. These mice were maintained in a barrier-system. Animals in the barrier-system were kept at 22 ± 1°C with a humidity of 55 ± 5% under a lighting regimen of 12 L:12 D (lights on from 07:00 to 19:00).

Source of embryos: Female mice were superovulated with 5 IU of PMSG (Serotropin, Teikoku Hormone Mfg. Co., Japan) and 5 IU of hCG (Gonatropin, Teikoku Hormone Mfg. Co., Japan) injected 48 hr apart, and the oocytes were obtained 15–16 hr after the hCG injection. Spermatozoa were collected from the cauda epididymis of males and suspended in 300 μl of TYH medium [8], covered with mineral oil (Fisher Scientific, USA). After preincubation for 1.5 hr at 37°C under 5% CO₂ in air, a small volume of sperm suspension was added to the TYH medium containing oocytes (final sperm concentration: 150 cells/μl). Pronuclear oocytes (the criteria for fertilized oocytes were the presence of two pronuclei and a second polar body) and 2-cell embryos obtained 6 and 24 hr after insemination, respectively, were washed with Whitten’s medium [9] and then used for the vitrification experiments.

Cooling and warming: About 40 oocytes/embryos were first pretreated with PBI containing 1 M dimethyl sulfoxide (1 M DMSO solution) at room temperature. Then 5 μl of 1 M DMSO solution containing oocytes/embryos was transferred into a 1 ml cryotube (NUNC, Denmark). The samples were then placed in ice water for 5 min to allow DMSO to permeate the oocytes/embryos sufficiently, and 95 μl of a highly concentrated solution (DAP 213; 2 M DMSO, 1 M acetamide, 3 M propylene glycol) [4] at 0°C, was added to each cryotube (final concentration: 1.95 M DMSO, 0.95 M acetamide, 2.85 M propylene glycol). After standing it for 5 min, the samples were plunged directly into liquid nitrogen and stored for 1 to 5 days before warming. The samples were removed from the liquid nitrogen, warmed at room temperature for 30 to 60 sec, and diluted with 0.9 μl of PBI medium [11] containing 0.25 M sucrose. The contents of each tube were pipetted into a watch glass to recover the oocytes/embryos. The recovered oocytes/embryos were washed with 4 changes of PBI medium, 3 changes of Whitten’s medium, and the morphological appearance was recorded. In addition, in order to examine the tolerance to the damage during vitrification, BDF1 pronuclear oocytes were repeatedly cooled and warmed three times in the same way.

Viability of cryopreserved oocytes/embryos: Some of the morphologically normal cryopreserved oocytes/embryos were cultured in vitro for 80–96 hr, and the number of blastocysts recorded. The remainder were transferred to the oviducts of pseudopregnant recipients on the day of vaginal plugs (Day 0.5 of pseudopregnancy). The fetuses that developed were observed on Day 18.5 of pregnancy.

Table 1 shows the morphologically normal rate of the cryopreserved pronuclear oocytes and 2-cell embryos after warming. Nearly all of the cryopreserved oocytes/embryos were recovered, and most of them were morphologically normal.

Table 2 summarizes the rate development to blastocysts from cryopreserved oocytes/embryos. The proportion of cryopreserved oocytes/embryos developed to blastocysts was high (84.8 to 92.3%), and there was no significant difference in the percentage of blastocysts when compared with that of the fresh pronuclear oocytes (control).

Table 3 shows the development to live fetuses from cryopreserved oocytes/embryos. The rate of development of 2-cell embryos cooled and warmed only once and pronuclear oocytes cooled and warmed three times repeatedly into live fetuses was 56.5% and 43.2%, respectively. There was no significant difference in the rate of survival to live fetuses between the cryopreserved...
Table 2. *In vitro* development of cryopreserved pronuclear oocytes and 2-cell embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>Strain</th>
<th>No. of oocytes/embryos</th>
<th>Cultured</th>
<th>Developed to blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pronuclear oocyte</td>
<td>C57BL/6J</td>
<td>115</td>
<td>102</td>
<td>88.7</td>
</tr>
<tr>
<td>2-cell</td>
<td>C57BL/6J</td>
<td>39</td>
<td>36</td>
<td>92.3</td>
</tr>
<tr>
<td>Pronuclear oocyte*</td>
<td>BDF1</td>
<td>66</td>
<td>56</td>
<td>84.8</td>
</tr>
<tr>
<td>Control**</td>
<td>C57BL/6J</td>
<td>161</td>
<td>144</td>
<td>89.4</td>
</tr>
</tbody>
</table>

* Pronuclear oocytes were repeatedly cooled and warmed three times.
** Control: fresh pronuclear oocytes.

Table 3. Development *in utero* of cryopreserved pronuclear oocytes and 2-cell embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>Strain</th>
<th>No. of oocytes/embryos</th>
<th>Transferred</th>
<th>Developed to live fetuses (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-cell</td>
<td>C57BL/6J</td>
<td>276</td>
<td>156</td>
<td>56.5</td>
</tr>
<tr>
<td>Pronuclear oocyte*</td>
<td>BDF1</td>
<td>74</td>
<td>32</td>
<td>43.2</td>
</tr>
<tr>
<td>Control**</td>
<td>C57BL/6J</td>
<td>55</td>
<td>30</td>
<td>54.5</td>
</tr>
</tbody>
</table>

* Pronuclear oocytes were repeatedly cooled and warmed three times.
** Control: fresh 2-cell embryos.

2-cell embryos and fresh 2-cell embryos (control). However, the survival of pronuclear oocytes cooled and warmed repeatedly three times was significantly lower than that of the control (P<0.05).

It was reported that mouse pronuclear oocytes and 2-cell embryos can be cryopreserved successfully by directly plunging them into liquid nitrogen, immediately after the exposure to a highly concentrated cryopreservation solution (ultrarapid freezing method). By this method, 58–73% of the morphologically normal oocytes/embryos developed to blastocysts [3, 4]. Nevertheless, the survival of embryos frozen and thawed repeatedly by the ultrarapid freezing method increased the number of damaged embryos. Therefore, the rate of morphologically normal embryos after the embryos were repeatedly frozen and thawed three times was very low (<30%, unpublished observation). In the present study, the rate of development to blastocysts of cryopreserved oocytes/embryos was higher than that in the study reported previously. Moreover, most pronuclear oocytes repeatedly cooled and warmed three times were morphologically normal and some of them developed to live fetuses. In addition, even the inbred strain C57BL/6J 2-cell embryos repeatedly cooled and warmed three times survived successfully, and developed normally *in vivo* like BDF1 pronuclear oocytes cooled and warmed three times did (data not shown).

Zhu *et al.* reported that when expanded blastocysts had been pretreated in 10% or 20% ethylene glycol solution for 5 min, followed by short exposure (0.5 min) to vitrification solution (40% ethylene glycol, 30% ficoll, 0.5 M sucrose) before plunging into liquid nitrogen, the post-warming survival rate increased to 83–84% [9]. Based on these experimental results, they asserted that the expanded blastocysts can survive vitrification if the toxic action of the vitrification solution is avoided, and sufficient cryoprotectant permeates the embryos before vitrification.

In our study, we first suspended the oocytes/embryos in a 1 M DMSO solution for 5 min before putting the DAP213 into a cryotube containing the oocyte/embryos. The exposure of oocytes/embryos to the 1 M DMSO solution may decrease the direct toxic action of the DAP213, because of the permeation of the oocytes/embryos by DMSO.

This novel simple vitrification method, presented here
is very simple. The oocytes/embryos can be handled easily during each step of the cooling and warming procedures. A high survival rate of mouse embryos is obtained after warming. This method is therefore recommended as a routine method for the cryopreservation for mouse embryos in various laboratories.

References