Efficacious cryopreservation of 2-cell stage mouse embryos via unvitrified ultrarapid freezing

Sadahiro Azuma

Department of Laboratory Animal Science, Kitasato University School of Medicine

Background: It has generally been accepted that mouse pre-implantation embryos can be preserved at -196°C in a vitrified suspension, thereby preventing water from crystallizing by transforming it into a glass instead. Whereas ultrarapid freezing is critical for the efficient cryopreservation of the embryos, vitrification might not be essential.

Objective: The processes for thawing and recovery of the embryos were modified to assess efficacy of cryopreservation in an unvitrified state.

Materials and Methods: Two-cell stage mouse embryos were suspended in a phosphate buffered medium (PB1) containing 1M dimethyl sulfoxide (DMSO) and transferred into a cryotube. The tubes were cooled to 4°C, at which point a non-vitrification medium was added (DP23; 2M DMSO, 3M propylene glycol). The tubes were then plunged into liquid nitrogen and stored. To thaw the embryos, the tubes were quickly transferred into a water bath at 37°C. The thawed medium containing the embryos was diluted by adding 900 μl of pre-warmed PB1 containing 0.3 M sucrose.

Results: Most of the recovered embryos (93.8%) were morphologically normal even after they were processed by ultrarapid freezing in DP23. A total of 58.3% of the embryos cryopreserved in DP23 developed in vivo until 17.5 dpc (days post coitum) when they were transferred into the oviducts of recipient female mice.

Conclusion: Two-cell stage mouse embryos can be cryopreserved by ultrarapid freezing in the unvitrified state, and quick warming is rather a decisive process.

Key words: mouse embryos, vitrification, cryopreservation, ultrarapid freezing, unvitrification solution

Introduction

Since Whittingham et al.1,2 first succeeded in cryopreserving mouse embryos in 1972, there has been remarkable progress in both basic and applied research enabling us to preserve the embryos of at least 13 more mammalian species.3,4 Moreover, every process used for freezing and thawing embryos has been refined and extremely simplified. Specifically: 1) The time required for the embryo to progress from freezing to storage temperature5-7 has been significantly shortened. 2) Sucrose was added to the cryoprotectant solutions to osmotically dehydrate the embryos prior to cooling.8,9 Sucrose also reduces the osmotic swelling that occurs during the dilution of the cryoprotectants.10 3) Immediately after warming and thawing, recovered embryos were transferred into either the oviducts or uteri of recipient females,11 and 4) Embryos were vitrified during the freezing and thawing procedures.12,13

Vitrification provides three important advantages over the conventional methods of embryo cryopreservation. Firstly, embryos in a vitrified state do not suffer tissue damage caused by ice crystallization during cooling, cryopreservation, and storage. Secondly, the vitrification procedure significantly shortens the time course to achieve cryopreservation. And thirdly, vitrification does not require specialized equipment. To date, it has been common knowledge that vitrification was absolutely necessary when embryos are cryopreserved by ultrarapid freezing.

In the present study, the survival rates of 2-cell stage mouse embryos that had undergone ultrarapid freezing with or without vitrification were examined to explore the bona fide effects of vitrification on recovery of the embryos. The ratios of the numbers of embryos recovered from unvitrified or vitrified cryopreservation were
compared paying particular attention to the different developmental stages in both the \textit{in vitro} and the \textit{in vivo} procedures. As a result, vitrification turned out to be sufficient but not essential for the cryopreservation of mouse embryos via ultrarapid freezing.

\textbf{Materials and Methods}

\textit{Embryos}

ICR female mice (8 – 16 weeks old; CLEA Japan, Tokyo) were superovulated by intraperitoneal injections of 7.5 IU of pregnant mare’s serum gonadotropin (PMSG, Peamex; Novartis Animal Health, Tokyo) and 7.5 IU of human chorionic gonadotropin (hCG, Puberogen; Novartis Animal Health, Tokyo) at 48-hour intervals. The oocytes were obtained approximately 15 – 16 hours following the hCG injection. Spermatozoa were collected from the cauda epididymis of ICR male mice (12 – 20 weeks old; CLEA Japan, Tokyo) and suspended into 200 μl of TYH medium\textsuperscript{14} covered with mineral oil (Fisher Scientific, USA). After pre-incubation for 1.5 – 2 hours at 37°C under 5% CO \textsubscript{2} in air, a small volume of sperm suspension was added to the TYH medium containing oocytes (final sperm concentration, 150 sperm/μl). Following incubation for 6 hours, the oocytes displaying 2 pronuclei after extrusion of the second polar bodies were taken out of the TYH medium. The fertilized oocytes were washed and cultured in 200 μl droplets of Whitten’s medium (WM; supplemented with 100 μM EDTA [ethylenediaminetetraacetic acid])\textsuperscript{15} under mineral oil at 37°C in an atmosphere of 5% CO\textsubscript{2} and air. After incubation for 22 to 24 hours, embryos that had progressed to the 2-cell stage were selected for cryopreservation.

\textit{Cryopreservation of mouse embryos}

Embryos were suspended at room temperature in a phosphate buffered medium (PB1) containing 1M dimethyl sulfoxide (Dojindo Laboratories, Kumamoto; 1M DMSO solution).\textsuperscript{16} Subsequently, 5 μl of the 1M DMSO solution containing embryos was transferred into a 1 ml cryotube (NUNC, Denmark). The tube was then put in a pre-cooled (4°C) metal block cooler (Chill Heat, CHT-101; Iwaki, Tokyo) for 5 minutes. Following cooling down, 95 μl (pre-cooled) of either DAP213\textsuperscript{17,18} (vitrifying solution; 2M DMSO, 1M acetamide; 3M propylene glycol; Figure 1A) or DP23 (crystallizing solution; 2M DMSO, 3M propylene glycol; Figure 1B) was added to the tube. After stabilization at 4°C for 5 minutes, the embryo suspension in the tube was plunged directly into liquid nitrogen and stored for 1 day up to 3 months before warming.

\textit{Recovery of cryopreserved embryos}

Mouse embryos frozen in DP23 were quickly warmed up via transferring of the cryotube from the liquid nitrogen container to a 37°C water bath. Cryoprotectants in the thawed embryo suspension were immediately diluted with pre-warmed 900 μl of PB1 containing 0.3M sucrose. In contrast, mouse embryos frozen in DAP213 were thawed at room temperature over a 30 – 60-second period, and cryoprotectant dilution was performed as described above. After thawing, the embryos were washed three times with PB1, rinsed another three times with WM, and eventually transferred to a culture medium to be analyzed for their viability and normality.

\textit{Viability and normality assay for cryopreserved embryos}

The recovered embryos displaying normal morphology continued to be cultured in 200 μl droplets of WM under mineral oil for 96 hours. The remaining embryos were transferred into the oviducts of pseudopregnant recipients on the day vaginal plugs were observed (Day 0.5 of pseudopregnancy). The recipient females were ICR mice that had been naturally mated with vasectomized males of the same strain (previously proven sterile). These females were autopsied on day 17.5 of gestation to

![A. A cryotube in which 2-cell stage mouse embryos were vitrified but not crystallized in DAP213 (2M DMSO, 1M acetamide; 3M propylene glycol). B. A cryotube in which the same stage mouse embryos are crystallized but not vitrified in DP23 (2M DMSO, 3M propylene glycol).](image-url)
determine the total number of both implantation sites and live fetuses in each uterine horn.

Statistical analysis
The obtained data was statistically analyzed using the \( \chi^2 \) test.

Results
To determine whether or not vitrification is necessary for cryopreservation of mouse preimplantation embryos upon ultrarapid freezing, I explored the details of its effects on the survival rates of the embryos after thawing. In this study, I used both vitrification and devitrification mediums, DAP213 and DP23, respectively, to compare towards the ultrarapid freezing of mouse 2-cell stage embryos. Notably, I utilized two distinct thawing methods for the cryopreserved embryos, depending on whether they were frozen with or without vitrification. I thawed the unvitrified mouse embryos by direct transfer of the cryotube into a 37°C water bath. In contrast, the vitrified embryos were warmed up at room temperature in advance for 30 to 60 seconds and quickly thawed when pre-warmed PB1 (containing 0.3M sucrose) was put into the cryotube to dilute cryoprotectants. Upon DAP213-mediated (vitrified) cryopreservation, 98.6% (276/280) of the embryos were recovered when they were thawed, and 93.8% (259/276) of these revealed normal morphology (Table 1). Unexpectedly, however, 99.1% (317/320) of the embryos processed by unvitrified cryopreservation in DP23 were recovered after thawing, and 93.7% (297/317) of these showed no morphological defects. Even in a preliminary analysis to test embryo recovery, the insignificant difference between the two mediums implies that vitrification may not be necessary for the preservation of embryos via ultrarapid freezing.

Therefore, I subsequently examined the recovered mouse embryos to determine whether or not those displaying normal morphology had suffered functional damage caused by freezing and thawing. I continued to culture the embryos in vitro until they progressed to the late blastocyst stage. The 2-cell stage embryos cryopreserved by ultrarapid freezing in a vitrified state (DAP213) gave rise to 4-cell, morula, early and late blastocyst stage embryos at rates of 98.5% (130/132), 92.4% (122/132), 77.3% (102/132), and 84.8% (112/132), respectively.

### Table 1. Survival rates of 2-cell stage mouse embryos cryopreserved at -196°C by ultrarapid freezing in an unvitrified medium

<table>
<thead>
<tr>
<th>Freezing medium</th>
<th>Vitrification</th>
<th>Frozen-thawed (No. of embryos)</th>
<th>Recovered (b/a, %)**</th>
<th>Survived (c/b, %)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAP213 +</td>
<td>280</td>
<td>276 (98.6)</td>
<td>259 (93.8)</td>
<td></td>
</tr>
<tr>
<td>DP23 -</td>
<td>320</td>
<td>317 (99.1)</td>
<td>297 (93.7)</td>
<td></td>
</tr>
</tbody>
</table>

**No significant differences were found between DAP213 and DP23 (P > 0.05).

### Table 2. Preimplantation development of in vitro mouse 2-cell stage embryos cryopreserved by ultrarapid freezing

<table>
<thead>
<tr>
<th>Freezing medium</th>
<th>No. of embryos cultured</th>
<th>No. of embryos developed to***:</th>
<th>4-cell (24 h)*</th>
<th>Morula (48 h)*</th>
<th>Blastocysts (72 h)*</th>
<th>(96 h)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAP213</td>
<td>132</td>
<td></td>
<td>130 (98.5)</td>
<td>122 (92.4)</td>
<td>102 (77.3)</td>
<td>112 (84.8)</td>
</tr>
<tr>
<td>DP23</td>
<td>144</td>
<td></td>
<td>142 (98.6)</td>
<td>139 (96.5)</td>
<td>120 (83.3)</td>
<td>128 (88.9)</td>
</tr>
<tr>
<td>Control**</td>
<td>108</td>
<td></td>
<td>108 (100)</td>
<td>106 (98.1)</td>
<td>90 (83.3)</td>
<td>95 (88.0)</td>
</tr>
</tbody>
</table>

*Hours after starting warm-up to thaw
**Control represents preimplantation development in vitro without freezing and thawing processes at the 2-cell stage.
***No significant differences were found between treatments (P > 0.05).
Efficacious cryopreservation of 2-cell stage mouse embryos via unvitrified ultrarapid freezing

respectively (Table 2). Similarly, even embryos frozen ultrarapidly in an unvitrified state (DP23) gave rise to normally developed 4-cell, morula, early and late blastocyst stages at the ratios of 98.6% (142/144), 96.5% (139/144), 83.3% (120/144), and 88.9% (128/144), respectively (Table 2). These observations suggest that, although vitrification of embryos in ultrarapid freezing allows us to freeze them with almost no tissue damage, it is unnecessary when we apply particular conditions for embryo freezing and thawing.

To further determine whether or not the 2-cell stage mouse embryos became functionally compromised as a result of cryopreservation upon ultrarapid freezing, I immediately transferred the thawed embryos into the oviducts of pseudopregnant recipient female mice. On day 17.5 following embryo transfer, I dissected the uteri of the recipient females and counted the number of implantation sites as well as morphologically normal fetuses. As a control for in vivo development, I examined 2-cell stage mouse embryos that had not undergone the freezing and thawing processes before being transferred to the oviducts of the recipients. In my experimental conditions, 74.5% (76/102) of the transferred 2-cell stage embryos implanted properly to the uteri, and 59.8% (61/102) of those developed into normal 17.5 dpc fetuses (Table 3). Similarly, of the embryos recovered from ultrarapid freezing in both the vitrified and unvitrified state and transferred to the oviducts, 75.8% (91/120) and 78.5% (113/144) exhibited normal implantation, respectively (Table 3). Furthermore, in spite of whether the embryos had undergone vitrified or unvitrified cryopreservation, 56.7% (68/120) and 58.3% (84/144), respectively, of the transferred embryos retained the potential to develop normally into 17.5 dpc fetuses (Table 3). These results proved that vitrification is sufficient but not necessary for cryopreservation of mouse preimplantation embryos via ultrarapid freezing if they are treated under particular conditions such as ultrarapid freezing in DP23 followed by quick thawing in a 37°C water bath.

Discussion

In the present study, I showed that 2-cell stage mouse embryos could be cryopreserved at a high recovery rate by plunging the cryotube directly into liquid nitrogen, even in the crystallizing condition. The cryopreserved and thawed embryos developed in vitro into blastocysts as well as in vivo into live 17.5 dpc fetuses after embryo transfer to the uterus of pseudopregnant female mice.

The mouse is the first mammalian species whose preimplantation embryos have been successfully cryopreserved.1,2 In the years since the first mouse embryo was successfully cryopreserved, both biochemical and biological studies have made significant contributions to mitigate the deleterious effect associated with each step of the procedure. In particular, the idea that embryos need to be cooled down and warmed up slowly to avoid tissue damage has been a matter of debate. Whittingham et al.,1 who originally succeeded in mouse cryopreservation, suspended embryos in saline containing a cryoprotectant at a 1M concentration. To freeze the embryos, the temperature was decreased to -78°C very slowly (0.3°C to 2°C per minute), after which they were transferred to liquid nitrogen for storage. They also raised the temperature slowly (4°C to 25°C per minute) to room temperature to thaw the embryos. Five years later, however, Willadsen et al.5 demonstrated that a slow cool-down was not even necessary once the temperature had fallen below -35°C. Their studies also revealed that the embryos could survive even if they were thawed rapidly. These alterations in the cooling and warming processes resulted in a significant reduction in the time needed for cryopreservation. We have currently made use of this

<table>
<thead>
<tr>
<th>Freezing medium</th>
<th>No. of embryos transferred</th>
<th>No. (%) of implantation sites**</th>
<th>No. (%) of live fetuses**</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAP213</td>
<td>120</td>
<td>91 (75.8)</td>
<td>68 (56.7)</td>
</tr>
<tr>
<td>DP23</td>
<td>144</td>
<td>113 (78.5)</td>
<td>84 (58.3)</td>
</tr>
<tr>
<td>Control</td>
<td>102</td>
<td>76 (74.5)</td>
<td>61 (59.8)</td>
</tr>
</tbody>
</table>

*Percentage (%) represents survival rates of either implanted or normal fetuses which were developed from 2-cell-stage embryos and transferred to recipient females after the freezing and thawing processes.

**No significant differences were found between treatments (P > 0.05).
finding as a common protocol for preserving the preimplantation embryos of mice, as well as those of domestic animals and even humans. Of notes, 2-step freezing as well as sucrose-mediated cryoprotection of mouse embryos enabled us to further shorten the time required for embryo freezing and thawing.

It has been highlighted by recent studies that a direct transfer of embryos into liquid nitrogen enables embryos to be frozen rapidly to -196°C, which avoids us having to cool them over a long period of time. The first successful attempt at cryopreservation via rapid freezing was achieved with 8-cell and 16-cell stage mouse embryos. Embryos were suspended in 4M glycerol solution containing 0.25M sucrose as a cryoprotectant and frozen rapidly in a 0.5-ml plastic insemination straw by exposure to liquid nitrogen vapor. However, it should be noted here that, even after embryos were vitrified upon rapid freezing in the same condition and in the same process, they were recovered at a survival rate of 40% - 78% (Azuma, unpublished).

Nakagata et al. were the first to demonstrate that 2-cell stage mouse embryos can be cryopreserved at a high recovery rate even if ultrarapid freezing was used. In their study, embryos were frozen by plunging them directly into liquid nitrogen. Immediately before preceding their immersion into liquid nitrogen, they suspended the embryos in a vitrification solution (DAP213). Consequently, 58% - 73% of the embryos were recovered displaying normal morphology and developed into blastocysts. To improve the efficacy of the cryoprotection, Nakao et al. gave the embryos an additional pretreatment. The embryos were suspended in 1M DMSO solution for 5 minutes prior to DAP213 exposure and then plunged directly into liquid nitrogen. This method resulted in a more than 20% increase in a survival rate of the embryos following the freezing and thawing process. Vitrification renders water incapable of crystallizing into ice, making possible the rapid cooling of embryos down to -196°C without damage to embryonic tissues. Vitrification is alternatively defined as solidification—a physical process in which a liquid becomes vitreous in a solution as a result of an increase in cryoprotectant density as the temperature is lowered. The solid, called a glass, retains the normal molecular and ionic distributions of the liquid state and is, therefore, usually considered to be an extremely viscous, supercooled liquid. The concentrated intracellular and residual extracellular solutions were supercooled upon subsequent rapid cooling to -196°C leading to their transformation into metastable glasses. However, whether or not vitrified embryos can be successfully recovered depends on the ability to control changes in vitreous cellular cytoplasm within the embryonic tissue during the warming process. Warming should not be so slow as to devitrify the glassy cytoplasm, which leads to crystallization and subsequent significant tissue damage within the embryos. Thus, Rall et al. used a rapid warming strategy to thaw the vitrified embryos, transforming the glass-like substance within the cells into a liquid before devitrification and crystallization could occur. High rates of vitrified embryo survival were achieved due to the particular change in the warming strategy.

In the present study, we demonstrated a simplified procedure for the cryopreservation of 2-cell stage mouse embryos, namely ultrarapid freezing and thawing, resulting in a high survival rate of the embryos. It should be noted here that: 1) The freezing medium, DP23, used in this study is a non-vitrifiable freezing solution containing DMSO and propylene glycol as cryoprotectants; 2) Medium equilibration was carried out step-wise at room temperature down to 4°C; and 3) DMSO and propylene glycol were immediately removed from the embryonic tissues by rapid dilution with a sucrose solution. I believe that these improvements of the processes will serve to accelerate the development of cryopreservation with the ultimate objectives supporting human reproduction as well as the preservation of rare mammalian species.

Acknowledgements
The author thanks the staff at the Center for Genetic Studies of Integrated Biological Functions, Kitasato University School of Medicine, for their animal care, especially Takahashi A for technical assistance and management and Shibuya T for recipient care and technical assistance. This study was supported, in part, by the High-Tech Research Center of the Japanese Ministry of Education, Culture, Sports, Science and Technology.

References
Efficacious cryopreservation of 2-cell stage mouse embryos via unvitrified ultrarapid freezing


