Evaluation of Developmental Competence of Vitrified-warmed Early Cleavage Stage Embryos and their Application for Chimeric Mouse Production

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Abstract: The developmental competence of in vitro cultured embryos vitrified-warmed at an early cleavage stage (2- or 4, 8-cell stage) was examined by both direct transfer into recipient animals and after in vitro manipulation for chimeric mice production using embryonic stem (ES) cells. Vitrified-warmed embryos transferred at the morulae and blastocyst stages showed fetus development comparable to control embryos, although blastocyst development of vitrified-warmed embryos was significantly slower than that of controls. When vitrified-warmed early cleavage stage embryos were used for chimeric mouse production using ES cells, 1 to 10% of the injected or aggregated embryos developed into chimeric neonates and germ-line chimeric mice were obtained from all ES cell lines. This study indicates that embryos developed in vitro from vitrified-warmed embryos have equivalent competence with unvitrified embryos irrespective of stage of vitrification and that these vitrified-warmed embryos maintain adequate viability even after in vitro manipulation such as aggregation and microinjection with ES cells.

Key words: chimeric mice, early cleavage stage embryos, vitrification

The primary purposes of embryo cryopreservation are to establish an embryo bank for efficient maintenance of genetic resources for animal research and a stock of animal colonies in case of environmental accidents, genetic contamination or infectious disease, and for easy transport of animals from one place to another with less danger of infection [13, 14].

The invention of vitrification methods [17, 19] in which embryos and gametes are directly immersed in liquid nitrogen makes cryopreservation quicker and cheaper than the slow-freezing procedure. Recently, cryopreserved embryos were shown to be a good source of embryo manipulation in vitro, having been used to produce transgenic mice by injection of exogenous DNA into the pronuclear stage embryos [7, 10, 15] and by injection of embryonic stem (ES) cells into blastocysts.
The advantage of using cryopreserved embryos for in vitro manipulation is that the number of manipulated embryos can be predetermined based on the amounts of transformed ES cells and exogenous DNA and the number of recipient mothers for embryo transfer [16].

Obtaining in vivo and in vitro produced blastocysts takes a long time (>1 week) from the beginning of hormonal treatment of animals. The yield of 2-cell stage embryos by oviduct flushing, as was used by Nakao et al. [18], in which blastocysts cultured from 2-cell stage embryos were vitrified-warmed for microinjection of ES cells, is generally better than that of in vivo produced 8-cell or blastocyst stage embryos obtained by oviduct/uterus flushing. Therefore, collection followed by cryopreservation of early stage embryos is useful for embryo manipulation. In addition, most mouse embryos are currently cryopreserved at early stages and are not used for in vitro manipulation immediately after thawing. Thus, it is advantageous if cryopreserved embryos can be manipulated in vitro after thawing and culturing in vitro without reducing developmental competence.

In this study, embryos vitrified-warmed at an early cleavage stage (2-cell or 4, 8-cell stage) were examined for their developmental ability to morulae and blastocyst stages in vitro and subsequent fetus development after embryo transfer (ET). Chimeric mouse production was also tested to evaluate development of morulae and blastocysts derived from vitrified-warmed embryos after in vitro manipulation of aggregation and microinjection with ES cells.

Inbred C57BL/6J mice were obtained either from Japan SLC, Inc., (Shizuoka, Japan) or animal colonies maintained in our institute, and were kept at 22 ± 2°C under a lighting regimen of 12 L: 12 D (lights on from 07:00 to 19:00). All animals were treated according to the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Safety and Ethical Handling Regulations Committee for Laboratory Animal Experiments, the National Institute of Radiological Sciences, Japan. Females of age 8 to 12 weeks were primed with 5.0 IU of equine chorionic gonadotropin (eCG, Serotropin®, Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan). Forty-six to forty-eight hours later, 5 IU of human chorionic gonadotropin (hCG, Gonatropin®, Teikoku Hormone) was injected and the females were mated overnight with 10–24 week-old males. Between 46 and 48 h after the hCG injection, 2-cell embryos were collected by flushing the oviducts of the mated females with KSOM containing half strength MEM amino acids [1, 5, 9].

First, in vitro developmental competence of vitrified-warmed embryos to the morulae/blastocyst stage and fetal development after ET were examined. The 2-cell stage embryos from individual animals were equally distributed into three groups of 9–20 embryos (controlled pooling) [12]. The first group was the unfrozen control and was cultured for 40 h before ET. The second group of embryos was vitrified immediately after collection at the 2-cell stage, warmed 30 min later and cultured for 40 h before ET. The last group of embryos was cultured for 18 h in vitro and vitrified-warmed at the 4 or 8-cell stages followed by another 22 h of culture before ET. Because developmental speed was shown to reflect the developmental competence of embryos cultured in vitro [2, 3, 8, 11], blastocyst formation after 40 h culture was compared among treatments. All embryo cultures were conducted using KSOM containing amino acids in 5% CO2, 5% O2 and 90% N2 at 37°C. The vitrification and warming procedures were as described elsewhere [17]. Parts of the in vitro cultured embryos (9 out of 17 replicates) were transferred into the uterine horns of recipient female MCH:ICR mice (CLEA JAPAN, Inc., Tokyo, Japan) on day 3 of pseudopregnancy (day 1 is defined as the day of vaginal plug formation). Fetal development was examined on day 19. The development data shown as percentages were subjected to Tukey-Freeman arcsine transformation [21] for two-way ANOVA with groups of embryos from the same origin defined as a block. Multiple comparisons were carried out using the test of the least significant difference.

For chimeric mouse production, the culture period of vitrified-warmed early cleavage stage embryos was extended, if necessary, to obtain the proper stage of embryos. For ES cell injection, the warmed embryos vitrified at the 2-cell stage were cultured to the blastocyst stage for 50–60 h, and the 4, 8-cell stage embryos were cultured for 32–42 h. For aggregation, vitrified embryos were used one hour after warming (4, 8-cell stage embryos), or 18 h after warming (2-cell stage embryos). The ES cell line used in this experiment was the R1 cell line (a kind gift from Dr. A. Nagy at...
Table 1. Development of embryos vitrified-warmed at 2- or 4, 8-cell stage

<table>
<thead>
<tr>
<th>Stage of vitrification</th>
<th>Total no. of embryos (n)</th>
<th>Embryos (%h ± S.E.M.) with normal morphology after warming</th>
<th>Embryos (%i ± S.E.M.) developed to morulae</th>
<th>blastocysts</th>
<th>No. of morulae and blastocysts transferredd</th>
<th>No. (%j ± S.E.M.) of implantation site</th>
<th>fetus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controlf</td>
<td>211</td>
<td>–</td>
<td>105 (46 ± 5)</td>
<td>103 (53 ± 5)</td>
<td>96</td>
<td>73 (77 ± 7)</td>
<td>49 (53 ± 9)</td>
</tr>
<tr>
<td>2-cell stageg</td>
<td>212</td>
<td>187 (87 ± 3)</td>
<td>118 (62 ± 4)</td>
<td>61 (33* ± 5)</td>
<td>83</td>
<td>65 (77 ± 7)</td>
<td>47 (55 ± 7)</td>
</tr>
<tr>
<td>4, 8-cell stageh</td>
<td>212</td>
<td>192 (92 ± 4)</td>
<td>117 (60 ± 4)</td>
<td>73 (39* ± 4)</td>
<td>83</td>
<td>61 (75 ± 9)</td>
<td>46 (54 ± 11)</td>
</tr>
</tbody>
</table>

*Total of seventeen replicates of experiments. *Percent of total embryos (n). *Percent of surviving embryos except control that was shown as percent of total embryos (n). *A total of nine replicates of experiments. *Fetal development was examined on day 19 of gestation. 

The Samuel Lunenfeld Research Institute of Mount Sinai Hospital, Univ. of Toronto, Canada. The ES cells were cultured on the feeder cells in Dulbecco’s modified Eagle’s medium (code: 05919, Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) containing 20% fetal calf serum (FCS, JRH Biosciences, Inc., Lenexa, KS, USA), 2-mercaptoethanol, nucleosides, non-essential amino acids and leukemia inhibitory factor [22]. Transformation and selection of ES cell colonies were done as described previously [4]. The genes M, S, T, PC26, PC83 and PC107 were all constructed for targeted mutation. Trypsinized ES cells were cultured on a gelatin-coated petri dish for 30 min to remove feeder cells. The cell suspension was then carefully recovered and centrifuged, and the concentration of ES cells was adjusted to 0.5–1.0 × 10⁶ cells/ml with the FHM medium [1, 9] containing 20% FCS. The microinjection procedure was essentially the same as described elsewhere [6]. Each blastocyst was injected with 10–15 ES cells in FHM containing half strength MEM amino acids [1, 5] with Narishige microinjectors equipped with a piezo-micromanipulator system (Prime Tech Ltd., Ibaraki, Japan). After a recovery period of a few hours, the injected blastocysts were transferred into recipient females as described above. For the aggregation method, the zona pellucida was removed by brief exposure of embryos to acidic Tyrode’s solution. The zona-free embryos were cocultured with 10–15 ES cells in the microwells made by the aggregation needle (BLS®, Budapest, Hungary) in a 100 μl drop of KSOM with 20% FCS. The embryo-ES cell aggregates were moved to KSOM and cultured for 24 h before ET.

Table 1 shows in vitro developmental competence of vitrified-warmed embryos and fetal development after ET. Similar percentages of recovery and survival were observed between embryos vitrified at the 2- and 4, 8-cell stages. The in vitro development of both groups of vitrified-warmed early cleavage stage embryos to blastocysts (33 and 39%) was significantly delayed compared with control embryos (53%). However, this delay in development did not correlate with the development after ET; percentages of the implantation site and fetal development after ET were not significantly different among the three groups.

Speed of development as indicated by blastocyst formation seems to be compromised by the freezing process. The timing of cleavage division, especially the third cleavage division, is a possible noninvasive method of estimating embryo viability [2, 3, 8, 11]. Delayed blastocoel formation of vitrified-warmed embryos did not reflect fetal development (Table 1), indicating that cleavage divisions later than the third division are unlikely to reflect viability of embryos.

Because the first experiment showed that developmental competence of vitrified-warmed embryos was equivalent to control unvitrified embryos, we further evaluated the ability of these embryos for chimeric mouse production by both aggregation and injection methods. After in vitro culture of vitrified-warmed embryos for 60 h (2-cell stage embryos) or 42 h (4, 8-cell stage embryos), more than 90% of embryos developed to blastocysts that could be utilized in microinjection (data not shown). Seven to thirty-four percent of injected or aggregated embryos developed to neonates and 1 to 10% of these were chimeric mice (Table 2). Chimeric mice were mated at 8 weeks old.
with mature C57BL/6J mice to determine germ-line transmission. When offspring of these chimeric mice were agouti, polymerase chain reaction (PCR) and southern blotting were performed to confirm the existence of targeted mutations derived from ES cell lines (data not shown). The results showed that at least one germ-line chimeric mouse was obtained from each ES cell line tested. All germ-line chimeric mice were males and they have been successfully used as founders of a lineage of transgenic mice.

Since the aggregation method is simple and requires no sophisticated injection apparatus [20], this method is suitable for a small-scale laboratory. We have successfully applied this method to vitrified warmed 2- and 4, 8-cell stage embryos for producing chimeric mice (Table 2), indicating that vitrified-warmed early stage embryos can also be used with the aggregation method.

Mouse embryos are usually cryopreserved at stages between 2-cell and morulae. The embryo manipulation of cryopreserved mouse strains has to be postponed for a few months until the animals derived from cryopreserved embryos become mature enough for embryo collection. Together with the study by Nakao et al. [18], our study shows that embryos of the C57BL/6 strain may be cryopreserved at any stage of development for direct application to in vitro manipulation such as chimeric mouse production without compromising the outcome.

### Table 2. Production of germ-line chimeric mice by injection of nontransformed and transformed ES cells into blastocysts derived from vitrified-warmed early cleavage stage embryos

<table>
<thead>
<tr>
<th>Method of ES introduction</th>
<th>ES cell line</th>
<th>No. of treated embryos</th>
<th>No. (%) of newborn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>total chimeric mice [male:female]</td>
</tr>
<tr>
<td>Injection R1 (nontransformed)</td>
<td>70</td>
<td>12 (17) 4 (6) [4:0]</td>
<td>2</td>
</tr>
<tr>
<td>Aggregation R1 (nontransformed)</td>
<td>80</td>
<td>9 (11) 1 (1) [1:0]</td>
<td>1</td>
</tr>
<tr>
<td>Injection M (R1, transformed)</td>
<td>36</td>
<td>9 (25) 2 (6) [2:0]</td>
<td>2</td>
</tr>
<tr>
<td>Injection S (R1, transformed)</td>
<td>68</td>
<td>18 (26) 7 (10) [5:2]</td>
<td>1</td>
</tr>
<tr>
<td>Injection T (R1, transformed)</td>
<td>59</td>
<td>20 (34) 5 (8) [1:4]</td>
<td>1</td>
</tr>
<tr>
<td>Injection PC26 (R1, transformed)</td>
<td>102</td>
<td>7 (7) 1 (1) [1:0]</td>
<td>1</td>
</tr>
<tr>
<td>Injection PC83 (R1, transformed)</td>
<td>106</td>
<td>14 (13) 2 (2) [2:0]</td>
<td>1</td>
</tr>
<tr>
<td>Injection PC107 (R1, transformed)</td>
<td>118</td>
<td>12 (10) 5 (4) [5:0]</td>
<td>4</td>
</tr>
</tbody>
</table>

*For injection of ES cells warmed embryos were cultured for 50–60 h (2-cell embryos) or 32–42 h (4, 8-cell embryos). For aggregation, warmed embryos were cultured for 18 h (2-cell embryos) or 1 h (4, 8-cell embryos). ND: Not determined.

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### References