An Attempt of Cryopreservation of Mouse Embryos at the ACTREC Laboratory Animal Facility in India

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Abstract: Cryopreservation is the long-term storage of viable cells/tissue in liquid nitrogen. The present study was conducted to freeze 8-cell- to morula-stage mouse embryos from the ACTREC Laboratory Animal Facility using a "slow freezing and fast revival" method. In all, 4,088 embryos were collected from 495 donor female mice of ten different strains. An average recovery of 8 embryos per donor mouse were recorded. Of the 4,088 embryos, 3,946 embryos of normal morphology were frozen in 173 straws. They were cooled down using a controlled-rate freezing assembly, and the straws were directly plunged into liquid nitrogen for long-term storage. Out of these 3,946 frozen embryos, 2,650 were found to be viable after fast revival. The highest survival rate, 81%, was recorded in B6D2F1 hybrid mice, whereas the lowest rate, 51%, was recorded in the S/RV/Cri-ba mutant strain. Out of 2,650 viable embryos, 2,359 embryos (89%) developed to the blastocyst stage after 24 h of incubation in a CO2 incubator. The developed blastocysts were transferred surgically into 101 pseudopregnant female mice, of which 49 (48.5%) females were found to be pregnant. The highest percentage of pregnancy, 75%, was recorded in C57BL/6NCrl and NIH-III mice, whereas no pregnant recipients were recorded in Ptch, C3H/HeNCrl and NOD SCID mice. Based on the deliveries of these 49 females, an average of 4 young were delivered per female. Improvement in efficiency of freezing, thawing, and surgical transfer of embryos into pseudopregnant females is one of the challenges in such studies.

Key words: ACTREC, cryopreservation, mouse embryo, propanediol

Introduction

Maintaining various strains of laboratory mice including genetically modified (GM) mice requires great efforts and special attention for such things as routine checking of breeding performance, health status, genetic purity, genotyping, and record keeping. Moreover, once these strains have been used and no more work is planned with them for the immediate future, there are basically two options. One is to keep a few pairs under continuous mating, which requires precious space, time, and cost; the other option is to discard them and import new pairs whenever required [27]. Under these circumstances, it is extremely advantageous to cryopreserve germplasm of such strains for future use. A bank of cryopreserved embryos also assists the management of animal facilities by providing protection against loss from breeding failure, catastrophe, infectious disease outbreaks, or genetic drift [9, 18, 27]. Cryopreservation also avoids the logistics associated with the transport of live mice from one place to another [20]. Transport of animals require appropriate transport cages, proper environmental conditions, food, a water source, road connectivity, regulatory permission to transport the animals, and quarantine.

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Embryos from a mouse were first frozen using controlled slow cooling in 1972 by Whittingham [30]. Currently, controlled slow freezing, vitrification, and rapid freezing in liquid nitrogen vapor are the three techniques being used for cryopreservation of rodent embryos [15]. Controlled slow cooling is based on the principle of dehydration, in which the cooling rate is controlled to remove water from the embryo, preventing cryoinjury by formation of ice crystals [2, 3, 18]. The primary component in a successful freezing solution is cryoprotectant. Propanediol is one of the efficient cryoprotectants used for the freezing of the mouse embryos. It permeates rapidly into the blastomeres and also diffuses out rapidly during thawing [19]. It also limits the amount of intracellular ice formation after slow cooling of 8-cell mouse embryos. This property may explain the universal use of propanediol in cryopreservation of mouse embryos [10].

In the beginning, Whittingham et al. [33] reported cryopreservation of 8-cell mouse embryos using a slow freezing and fast thawing method. Uechi et al. have reported a low implantation rate for vitrification as compared with slow controlled-rate freezing [26]. Furthermore, Uechi et al. also warn that vitrification may exert more harmful effects than slow controlled rate freezing [26]. However, few studies in the recent past have reported simpler and quicker methods of vitrification [7, 8, 16]. The present study was planned at the Advanced Centre for Treatment, Research and Education in Cancer (ACTREC) Laboratory Animal Facility with the objectives to cryopreserve preimplantation embryos of mice strains for long-term storage so as to reestablish the colonies from cryopreserved embryos.

**Materials and Methods**

Animals and husbandry

Embryos from 6–8-week-old nulliparous mouse strains from ACTREC were used in the present study. Original stock of breeders of Crl:CD1(ICR) mice, Crl:CFW(SW) mice, C3H/HeN Crl mice, C57BL/6N Crl mice, NOD.CB17-Prkdc<sup>scid</sup>/NcrCrl (NOD SCID) mice, and Crl:NIH-Lyst<sup>b<sup>Ty</sup>Foxn1<sup>nu</sup>Btk<sup>xid</sup></sup> (NIH-III) Nude mice were obtained from the Charles River Laboratories, Wilmington, MA, USA. B6D2F1 mice are F1 hybrid bred at ACTREC from cross of female C57BL/6N Crl and male DBA/2NCrI mice procured from Charles River Laboratories. S/RV/Cri-ba is a mutant mouse found at ACTREC from Swiss mouse colony procured from National Institute of Virology, Pune, India. Original stock of breeders of Ptc<sup>h</sup> knockout mice were procured from Howard Hughes Medical Institute, Chevy Chase, Maryland, USA. Original stock of breeders of BALB/c/Crl mice were procured from Bhabha Atomic Research Centre, Mumbai, India. This study was duly approved by the Institutional Animal Ethics Committee (IAEC) of the ACTREC in accordance with the CPCSEA guidelines. All strains of mice were housed in an Individually Ventilated Animal Caging System (Citizen Industries, Ahmedabad, Gujrat, India) and provided with commercially available corn cob bedding material (Natgrit 406, Natural Organics, Satara, MS, India). The animals were housed in a controlled environment at 23 ± 2°C with 40–70% relative humidity and a 12-h/12-h dark/light cycle. The animals received sterile water ad libitum and autoclaved balanced diet prepared in-house from natural ingredients like wheat, roasted Bengal gram, casein, milk powder, ground nut oil, vitamins, and mineral supplements, which provided approx. 21% crude protein. Timed pregnant females were obtained by natural mating and checking for the presence of a vaginal plug (VP) the next morning.

Making pseudopregnant females

Male mice were sterilized by surgical removal of a piece of the vas deferens [5]. Young 6–8-week-old females were kept for mating with these sterile males after at least 2–3 weeks after vasectomy. Females observed to have a VP on the morning after mating were used as surrogate mothers for surgical transfer of blastocyst-stage embryos.

Embryo culture media

Commercially available media was procured from William A. Cook Australia Pty. Ltd., Brisbane, Australia (http://www.cookartlab.com/featured_products.php). The kit comprises a Sydney IVF Cryopreservation Kit (K-SICS-5000), Sydney IVF Thawing Kit (K-SITS-5000), and Sydney IVF Blastocyst Medium (K-SIBM-50). The K-SICS-5000 kit consists of three vials, namely, F1, F2, and F3, which contain cryopreservation buffer, cryopreservation buffer with 1.5 M propanediol, and 1.5 M propanediol plus 0.1 M sucrose, respectively. The K-SITS-5000 kit consists of four vials, namely, T1, T2, T3, and T4, which contain cryopreservation buffer with 1.0 M propanediol and 0.2 M sucrose, cryopreservation buffer with 0.5 M propanediol, cryopreservation
buffer with 0.2 M sucrose, and cryopreservation buffer, respectively. The K-SIBM-50 medium is a bicarbonate buffered medium used to culture of 8-cell- to morula-stage embryos to the blastocyst stage.

Collection of embryos

On day 2.5 post coital, VP-positive females were sacrificed by cervical dislocation to collect the 8-cell- to compacted morula-stage embryos. Both oviducts were excised along with a small piece of the adjoining uterus and placed in a drop of 50 µl of F1 (K-SICS-5000) solution in Petri dishes (Cat No. 150288; Nunc, Rochester, NY, USA). The oviducts were slowly flushed with F1 solution using a 30 G needle by holding the infundibulum under a stereomicroscope (model SMZ-800; Nikon, Tokyo, Japan) to expel the embryos. The embryos were siphoned, transferred to a fresh drop of F1 medium, and washed thrice with the same medium to get rid off the debris.

Criteria for selection of embryos

Eight-cells- to compacted morula-stage embryos were selected microscopically based on round form, normal size, normal cytoplasmic granulation and intact zona pellucida [27].

Freezing and thawing procedure

The freezing protocol used was based on those of Pomeroy [18], Uechi et al. [26] and the Jackson Laboratory (Bar Harbor, ME, USA, http://cryo.jax.org/slow.html). All clean embryos were transferred to F2 (K-SICS-5000) medium for 10 min of equilibration. After 10 min, the embryos were transferred in F3 (K-SICS-5000) medium for another 10 min. On average, 20–25 embryos were loaded into each labeled 0.25 ml capacity plastic straw (cat no. 006430; IMV Technologies, L’Aigle, France) using three columns of F3 medium separated by air bubbles from a central column containing the embryos as shown in Fig. 1A [1, 15]. The open ends of the straws were sealed with the help of a plastic sealing machine. Freezing was carried out in a programmable freezer (model CL-8800, CryoLogic Pty. Ltd., Victoria, Australia). Straws were inserted in a cryochamber in the vertical position, keeping the cotton plug at the upper side. Straws were cooled down from room temperature to −7°C at a rate of 2°C/min; the initial holding time was 5 min. Manual seeding was performed at −7°C by touching the cotton bud dipped in liquid nitrogen at the upper end of the central column of media containing embryos as shown in (Fig. 1A). Temperatures of straws were then dropped down to −30°C at a rate of

Fig. 1. (A) Schematic representation of the 0.25 ml capacity straw used for cryopreservation. A and D: F3 medium. B: Air bubble. C: Embryos in F3 medium. E: Sealed end. (B) Viable frozen-thawed embryos (thick arrows) along with a few cryoinjured mouse embryos (thin arrows). ×100. (C) Blastocysts with clear blastocoels (thick arrows) and inner cell masses (thin arrows) after culture for 24 h. ×200.
0.5°C/min and again from −30 to −120°C at a rate of 1°C/min. After holding them at −120°C for 5 min, the straws were directly plunged into liquid nitrogen for long-term storage.

Thawing was performed by quickly removing each straw from the liquid nitrogen, holding them in the air at 24°C for 40 s and then dipping them in a 37°C water bath for 30 s [14]. Immediately after thawing, the embryos were expelled into a Petri dish. All embryos were serially transferred from T1 to T4 (K-SITS-5000) medium to remove the cryoprotectant as well as sucrose from the embryos with 5 min of equilibration in each step. Postthaw survival was assessed based on the morphology of the embryos observed under a microscope. Subsequently, the surviving embryos were cultured in vitro by using Blastocyst Medium (K-SIBM-50).

**In vitro and in vivo survival**

Frozen-thawed embryos washed as above were cultured in a 75 µl drop of the Blastocyst Medium covered with paraffin oil (Cat. no. M-8410; Sigma-Aldrich, St. Louis, MO, USA) in tissue culture-grade Petri plates. These plates were kept in a CO₂ incubator (model 3111; Thermo Scientific, Waltham, MA, USA) at 37°C in 5% CO₂ for 24 h. Survival of the 8-cell- to morula-stage embryos was assessed by their ability to develop into fully expanded blastocysts with a blastocoel cavity. Twenty to twenty-five fully developed blastocysts were surgically transferred into the uterotubal junction of the left uterine horn in anesthetized 3.5-day-old pseudopregnant females [6].

**Statistical analysis**

Statistical analysis was performed using the Chi-square exact test.

<table>
<thead>
<tr>
<th>Results</th>
</tr>
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</table>

**Collection of embryos**

A total of 4,088 embryos from 495 donor females were collected; 3,946 of the embryos were morphologically normal as per the selection criteria. The remaining 142 (3.5%) embryos were from the 2–4 cell stage and were not included in this study. The CFW strain yielded 4.8% of such embryos, whereas BALB/c Cri yielded 16.9% of such embryos, which was significantly higher than the other strains. The average number of 8-cell- to morula-stage (freezable) embryos recovered per mouse was 8. In all, 173 straws were used to freeze the 3946 embryos. On average, 23 embryos were stored in each straw. A summary of the embryos collected and frozen by strain is presented in Table 1.

**In vitro and in vivo survival**

The overall rate of viable embryos (Fig. 1B) after rapid thawing was 67.2%. The rate of survival after thawing was found to be lower in a few inbred mice strains like S/RV-Cri ba (51%), NOD SCID (53.6%), C3H/HeNCri (55.4%), BALB/c/Cri (58.9%), and C57BL/6N Cri (60.8%) when compared with the average value of 67.2%. The best viability was observed in B6D2F1 mice (81.1%) followed by immunocompromised NIH-III Nude mice (74.8%), Ptch mice (69.8%), CD1 mice (68.1%), and CFW mice (67.9%). After 24 h. of incubation, the percentage of embryos that reached the blastocyst stage (Fig. 1C) ranged between 68.8 to 96.5% amongst all the strains. The NOD SCID, C57BL/6, CD1, and BALB/c strains yielded less than 90% in vitro survival, whereas more than 90% of the 8-cell/morula-stage embryos of strains NIH-III, C3H/HeNcri, Ptch, B6D2F1, CFW, and Swiss bare survived in vitro and reached the blastocyst stage. The percent of live pups born ranged from 7.5–17.4%. Ptch, C3H/HeNcri, and NOD SCID mice did not yield any live pups. We observed statistically significant interstrain differences with respect to recovery of viable embryos after thawing (P<0.0001), blastocyst development (P<0.0001), and live pups born (P=0.008). The data concerning viable embryos, subsequent development into blastocysts, and live fetuses born are summarized in Table 1.

**Discussion**

During the past decades, most research has demonstrated that frozen storage of mouse embryos has no detrimental effect on the subsequent ontogenic process [22]. In this study, the average gain of embryos per female by natural mating was found to be satisfactory. The genetic backgrounds of mice are a major factor for reproductivity and embryo recovery [4, 25]. In this study, CFW (SW) and BALB/c Cri mice always yielded different stages of embryos. This may be attributed to slower in vivo developmental rates than the other strains [21]. In practice, mouse embryos at any preimplantation stage can be frozen and revived successfully [28–30].
However, cryopreservation of 8-cell-stage embryos is advantageous because they are easy to access and assess morphologically, more robust, and respond well to freezing-thawing methods. The greatest advantage is that embryos at this stage permit postthaw viability to be accurately assessed by culture before transfer into recipient pseudopregnant females [20, 24, 29]. Therefore, in this study, we adopted freezing of 8-cell embryos.

For the present study, we used 1,2-propanediol as a cryoprotectant [13, 17, 23]. Renard and Babinet [19] reported a survival and blastocyst development rate of 80.8% with a high rate of thawing (~2,500°C/min), whereas they reported a survival and blastocyst development rate of 89.1% with a low rate of thawing (~300°C/min). Leibo and Mazur [11] demonstrated that the optimal freezing rate for mammalian embryos is approximately 0.2 to 2°C per min. Exposure time of embryos to the cryoprotectant strongly influences their viability after thawing. Therefore, the equilibration time in this slow freezing protocol was maintained as neither too short nor too long to avoid substitution of water molecules and the toxic effects of the cryoprotectant in the embryos, respectively [12].

Viability after thawing varied among the mutant and inbred strains of mice. The postthaw viability in the present study ranged from 52.1 to 81.1%. Increased sensitivity of embryos to the freezing and thawing process and type of cryoprotectant may be responsible for the varying range of viability [31]. In the present study, the rate of development to the blastocyst stage after in vitro culture of 8-cell- to morula-stage embryos for 24 h was found to range from 68.8 to 96.5%, which also corresponds to the earlier reports [1, 19].

The percentage of live fetuses born in the strains varied from 7.5 to 17.4%. The overall low success rate of live fetuses born in this study may be due to the synchronous transfer of blastocysts into the uterus. Asynchronous transfer of blastocysts to the oviduct yields better implantation than synchronous transfer [19]. In the case of transfer of blastocysts to C3H/HeNCrI, Ptch and NOD SCID mice, no pregnancy was found. This may be because of the very small sample size [32]. However, technical skill and strain differences cannot be ruled out. Further studies are required using a significant number of surgical transfers in these strains. Studies are also needed that target use of both horns of the uterus for embryo transfer. Pregnancies and birth of normal live fetuses from transfer of thawed embryos into the pseudopregnant females provided confirmation that our procedure is valid and effective.

In conclusion, the embryos that survive freezing and thawing are capable of developing to full term and producing normal, live young. Our results imply that storage of the embryos of mice that are not in current use but may be required in the future is possible. The stored embryos could be thawed and transferred to pseudopregnant females as per requirement, and the resulting offspring can be used to reestablish the nucleus of the re-

**Table 1. Results of cryopreservation of mouse embryos in ten mouse strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of females used</th>
<th>No. of embryos collected</th>
<th>No. of recovered embryos at the 2-4-cell stage**</th>
<th>No. (Mean ± SD) of embryos recovered at the 8-cell &amp; morula stage</th>
<th>Number of embryos frozen</th>
<th>No. of normal embryos after thawing</th>
<th>No. of embryos developed to blastocysts and transferred</th>
<th>Number of pseudopregnant females used</th>
<th>No. of pregnant recipients</th>
<th>Number of live births*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFW(SW)</td>
<td>162</td>
<td>1,326</td>
<td>63 (4.8)</td>
<td>8.2 ± 2.1</td>
<td>1,263</td>
<td>857 (67.9)</td>
<td>781 (91.1)</td>
<td>35</td>
<td>16 (45.7)</td>
<td>65 (8.3)</td>
</tr>
<tr>
<td>CD1(ICR)/Cri</td>
<td>79</td>
<td>845</td>
<td>5 (0.6)</td>
<td>10.7 ± 4.5</td>
<td>840</td>
<td>572 (68.1)</td>
<td>495 (86.5)</td>
<td>21</td>
<td>11 (52.4)</td>
<td>37 (7.5)</td>
</tr>
<tr>
<td>S/RV/Cri-ba</td>
<td>17</td>
<td>147</td>
<td>0</td>
<td>8.6 ± 1.5</td>
<td>147</td>
<td>75 (51)</td>
<td>68 (90.7)</td>
<td>4</td>
<td>02 (50)</td>
<td>06 (8.8)</td>
</tr>
<tr>
<td>B6D2F1</td>
<td>64</td>
<td>580</td>
<td>3 (0.5)</td>
<td>9.1 ± 4.1</td>
<td>577</td>
<td>468 (81.1)</td>
<td>433 (92.5)</td>
<td>16</td>
<td>10 (62.5)</td>
<td>38 (8.8)</td>
</tr>
<tr>
<td>BALB/c/Cri</td>
<td>50</td>
<td>419</td>
<td>71 (16.9)</td>
<td>8.4 ± 3.7</td>
<td>348</td>
<td>205 (58.9)</td>
<td>178 (86.8)</td>
<td>8</td>
<td>04 (50.0)</td>
<td>31 (17.4)</td>
</tr>
<tr>
<td>C57BL/6NCrI</td>
<td>37</td>
<td>217</td>
<td>0</td>
<td>5.9 ± 1.7</td>
<td>217</td>
<td>132 (60.8)</td>
<td>113 (85.6)</td>
<td>4</td>
<td>03 (75.0)</td>
<td>10 (8.8)</td>
</tr>
<tr>
<td>Ptch</td>
<td>10</td>
<td>63</td>
<td>0</td>
<td>6.3 ± 0.7</td>
<td>63</td>
<td>44 (69.8)</td>
<td>41 (93.2)</td>
<td>2</td>
<td>00 (0)</td>
<td>0</td>
</tr>
<tr>
<td>C3H/HeNCrI</td>
<td>15</td>
<td>101</td>
<td>0</td>
<td>6.7 ± 0.7</td>
<td>101</td>
<td>56 (55.4)</td>
<td>53 (94.6)</td>
<td>2</td>
<td>00 (0)</td>
<td>0</td>
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<tr>
<td>NIH-HII</td>
<td>20</td>
<td>151</td>
<td>0</td>
<td>7.6 ± 1.8</td>
<td>151</td>
<td>113 (74.8)</td>
<td>109 (96.5)</td>
<td>4</td>
<td>03 (75.0)</td>
<td>11 (10.1)</td>
</tr>
<tr>
<td>NOD SCID</td>
<td>41</td>
<td>239</td>
<td>0</td>
<td>5.8 ± 2.1</td>
<td>239</td>
<td>128 (53.6)</td>
<td>88 (68.8)</td>
<td>5</td>
<td>00 (0)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Calculated based on the no. of embryos transferred vs. the no. of live births. ** Nonfrozen embryos, not included in this study. Values in parentheses indicate percentages. Statistically significant interstrain differences of recovery of viable embryos (P<0.0001), blastocyst development (P<0.0001), and live birth (P=0.008). Embryos were collected at day 2.5 p.c. from the donor mice.
spective strains. We propose freezing of at least 500 embryos per strain to keep an embryo bank viable. Based on our current findings, freezing 500 embryos will yield a minimum of 13 live pups and a maximum of 68 live pups. Our experience suggests that delivery of a minimum 13 pups is sufficient to revive a strain. However, we need to improve the embryo gain, freezing, thawing, and surgical transfer protocol so that the rate of production of live born pups can be increased substantially. Since novel methods of equilibrium vitrification of mouse embryos have been reported in the recent past, in order to improve the success rate, our next target would be to use equilibrium vitrification of 2-cell-stage mouse embryos. Moreover, establishment of a multicentric embryo bank is the need of the hour and will help in guiding the research for improving the overall success of the cryopreservation technique and meeting the demands of the research community.

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