Transport of 2-cell embryos produced by intracytoplasmic sperm injection (ICSI) at cold temperatures (4°C)

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Abstract

Previously, we have succeeded in transporting vitrified/warmed 2-cell mouse embryos while maintaining developmental ability at refrigerated temperatures for 50 hours. In this study, we examined the developmental ability of 2-cell stage embryos produced by Intracytoplasmic Sperm Injection (ICSI), which were then refrigerated and transported. Sampling tubes containing 2-cell stage embryos produced by ICSI were placed into embryo transport containers and transported from the Institute of Advanced Technology, Kinki University, Wakayama to the Center for Animal Resources and Development (CARD), Kumamoto University, Kumamoto. The time required for transport was around 2 days. During transport, the temperature of the tubes was kept between 2.5 and 5.0°C. At CARD, the embryos were recovered from the sampling tubes and transferred into the oviducts of pseudopregnant females on the day that a vaginal plug was found (Day 1 of pseudopregnancy). The rate of viable 2-cell stage embryos after transport was 100% (122/122), of which 21% developed to live young.

1. Introduction

At present, a variety of genetically modified animals are produced (4), and the research as a human disease-model animal is advanced. There is an exceptionally large number of genetically modified mice and the number is on the increase (14). For this reason, the problems of breeding space and cost have recently arisen. Moreover, in order to supply genetically modified mice in response to requests from researchers, we urgently require the appropriate bio-resources, i.e. embryos and gametes (22). These issues are occurring all over the world.

The cryopreservation technology for mouse embryos was developed by Whittenghum, who succeeded in freezing them at -196°C using a slow freezing method for the first time (21). Thereafter the vitrification method of embryos was developed further by Rall (16). Rall’s method involves embryos being rapidly cooled to prevent ice crystal formation by exposing the embryo to a high density cryoprotective solution. Furthermore, as the number of genetically modified animals being transported increases, the transportation of these animals in and out of the country also increases. In order to prevent infections, accidents and inconveniences, more and more embryos are being frozen before being transported. However, the survival of the cryopreserved embryos depends on the storage temperature as well as the technique used (7). Thus, a standardized technique used for transporting embryos at cold temperatures has been widely employed (18). The technique reported allows early embryos to be transported at cold temperatures for up to 50 hours (19). Furthermore, additional techniques are not required to thaw the embryos, meaning that the embryos are ready to be transplanted soon after arrival. This transportation technique has also been developed for vitrified embryos and in vitro fertilized eggs using frozen sperm.

This study examined the transportation of embryos using assisted reproductive techniques, with focus placed on ICSI for the maintenance and supply of individual strains.
2. Materials and Methods

2.1 Animals
C57BL/6J mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and used as oocyte and sperm donors. Female donors were 8-12 weeks of age and male donors were 12 weeks of age. Simultaneously, MCH (ICR) mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and used as recipients for the transfer of 2-cell stage embryos at 8-16 weeks of age. All animals were kept under a 12h/12h dark/light cycle at a constant temperature of 23±2°C with free access to food and water. All animal procedures conformed to the Guidelines of Kinki University for the Care and Use of Laboratory Animals.

2.2 Embryo manipulation media
mCZB medium and mCZB-HEPES (10) medium was used for ICSI. These culture media were modified by referring to the medium produced by Chatot (3). KSOM medium (12) was used for embryo culturing after ICSI.

2.3 Cold transport and embryo transfer media
M2 medium (15) was used for the cold storage of 2-cell stage embryos. Next, modified Whitten’s medium (6) was used for embryo collection and embryo transfer after transport.

2.4 Intracytoplasmic sperm injection (ICSI)
Male mice were sacrificed via cervical dislocation, and cauda epididymides were collected. Sperm collected from the cauda epididymides were suspended in a 1.5mL tube containing 100µL of mCZB-HEPES medium. Thereafter, the sperm heads and tails were separated via sonification (2°), as only the sperm heads were used in ICSI. Female mice were induced to superovulate via an intraperitoneal injection of 7.5IU PMSG (Sankyo-yell Pharmaceutical Co., Ltd.) followed by an injection of 7.5IU hCG (ASKA Pharmaceutical Co., Ltd.) 48h later. At 14-16h after the injection of hCG, the mice were sacrificed via cervical dislocation and their oviducts were removed. Next, the cumulus-free oocytes were collected into a 200µL drop of mCZB-HEPES with 0.1% hyaluronidase from the oviduct ampulla. The procedure of micromanipulation was essentially the same as that described previously (11). A single sperm head was injected into the ooplasm with a minimal amount of accompanied medium. Once the sperm had been injected, viable oocytes were incubated in mCZB medium for 6h at 37°C with 5% CO2 in the air. These oocytes were observed for pronuclear formation, then transferred and cultured in KSOM medium (ARK Resource Co., Ltd) for 18h at 37°C with 5% CO2 in the air.

2.5 Embryo transport and Embryo transfer
For embryo transport, 2-cell stage embryos obtained by ICSI were placed in embryo transport containers (19), and transported from the Institute of Advanced Technology, Kinki University, Wakayama, to the Division of Reproductive Engineering, Center for Animal Resources and Development (CARD), Kumamoto University, Kumamoto. M2 medium (Sigma Co., Ltd.) was used to keep the embryos at 4°C during transport (Figure 1a, 1b, 1c). During transport, the temperature in each embryo transport box was continuously measured using a temperature data logger (COOL MEMORY; SANYO Electric Co., Ltd.). At the receiving institution, the embryos were washed in mWM (ARK Resource Co., Ltd) after being collected from the M2 medium. After washing, normally formed embryos were transferred to the oviducts of pseudopregnant females (5) on the day that a vaginal plug was found (Day1 of pseudopregnancy).

2.6 Statistical analysis
Statistical analysis in experiments of development rate into live youngs was performed by PLSD of Fisher using Computer software (Stat View 5.0). A significant difference was assumed to be P<0.05.
Figure 1. An example of cold transport method

a) 2-cell stage embryos produced by ICSI were gently moved M2 medium in 0.5mL tube. It was confirmed that 2-cell stage embryos was not remained in capillary. After, 0.5mL tube was stood in transport box. b) COOL MEMORY was continuously measured using a temperature data logger in each embryo transport box temperature. c) The transport box was packed in foam polystyrene. The foam polystyrene was stood in refrigerator until transportation. After, the foam polystyrene was transported at cold.

3. Results and Discussion

We performed ICSI using sperm heads taken from C57BL/6J male mice and MI oocytes of female mice, and developed them into 2-cell stage embryos. Table 1 shows the results of ICSI for C57BL/6J oocytes. The live rate after ICSI was 52% (205/392) and the yield rate of pronuclear stage oocytes was 73% (150/205). The development rate to 2-cell stage embryos was 83% (124/150). These results are in accordance with the results shown in previous reports (9).

Secondly, 2-cell stage embryos obtained by ICSI were land transported from the Institute of Advanced Technology, Kinki University to the Division of Reproductive Engineering, Center for Animal Resources and Development (CARD), Kumamoto University. As shown in Table 2, the 100% (122/122) of 2-cell stage embryos transported at cold temperatures were normal form upon arrival. This result was equal to that of vitrified/warmed 2-cell stage embryos transported at refrigerated temperatures (19).

During transport, the temperature in each embryo transport box was continuously measured using a temperature data logger. Figure 2 shows one example of the temperature distribution while the embryos were being transported. The temperature distribution showed a gradual decrease when the embryos were transferred from room temperature to refrigeration. Moreover, the transport temperature of the embryos (2.5-5.0°C) indicated distribution stability after 24h.

Finally, the embryos were retrieved from the M2 medium, then put into mWM and transferred to the oviducts of pseudopregnant females at the receiving institution. Table 3 shows the results of transfer of embryos obtained by ICSI after being transported. The incidence rate of live young derived from 2-cell stage embryos after transport at cold temperatures was 21% (26/122). This result was equal to the incidence rate of live young derived without transport at refrigerated temperatures (29%; 21/72). After, survival live youngs was weaned at 3 weeks from birth.

| Table 1. Fertilization of mouse oocytes by Intracytoplasmic Sperm Injection (ICSI) |
|-----------------|-----------------|-----------------|-----------------|
| No. of oocytes  | No. (%) of surviving oocytes | No. (%) of activated oocytes | No. (%) of developed to 2-cell stage embryos |
| 392             | 205 (52)         | 150 (73)         | 124 (83)        |

| Table 2. Viability of transported 2-cell stage embryos after Intracytoplasmic Sperm Injection (ICSI) |
|-----------------|-----------------|-----------------|
| No. of examined 2-cell stage embryos | No. (%) of viable 2-cell stage embryos after transport | No. (%) of morphologically normal embryos |
| 122             | 122 (100)       | 122 (100)       |
Table 3. Development of transported 2-cell stage embryos into live young

<table>
<thead>
<tr>
<th>Condition of transport</th>
<th>No. of 2-cell stage embryos transferred</th>
<th>No. of recipients</th>
<th>No.(%) of pregnancy</th>
<th>No.(%) of live youngs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refrigerate</td>
<td>122</td>
<td>5</td>
<td>5 (100)</td>
<td>26 (21)</td>
</tr>
<tr>
<td>Control</td>
<td>72</td>
<td>3</td>
<td>3 (100)</td>
<td>21 (29)</td>
</tr>
</tbody>
</table>

In conclusion, this study showed that it is possible to transport embryos fertilized via ICSI at refrigerated temperatures. We feel that the refrigerated transport method highlighted in this paper will become much more widely used in the future. The existing method of transporting early stage embryos consists of freeze preserving the embryos and sending them in a dry shipping container, which is costly in terms of shipping. In addition, it is necessary to return dry shipping containers to the sender, which also costs money. Finally, embryos transferred using dry shipping containers need to be thawed using a special method. The transportation method which we have developed requires no such thawing method, and so is a simple and low cost solution to this problem. Moreover, collection of the embryos shipped using our method is simpler than those using the dry shipping method.

Temperature descent speed was shown to be settled from 12 hour until 48 hour after refrigerated transport (Figure 2). This result was conformed to 50 hours after refrigerated transport of previous report. Accordingly, we enable to transport at refrigeration as Bioresource from Hokkaido aria until Kyusyu aria.

ICSI has been developed to assist fertilization, especially for mouse strains with low fertilization ability. Although ICSI is a powerful tool for fertilizing oocytes, it is difficult for beginners to master this technique. Therefore, if we can develop a system whereby a requester sends cryopreserved sperm with no motility to a specialist, who performs ICSI and returns fertilized embryos via the refrigerated transport method, the use of both ICSI and our refrigerated transport method will increase further in the future.

The refrigerated transport method for early stage embryos produced via ICSI outlined in this report is thought to be the first such report of its kind, and therefore contributes to the improvement of the supply system of early stage embryos obtained using assisted reproductive technology.

Figure 2. Result of temperature distribution during refrigerated transport.
4. Reference


和文抄録
卵細胞内精子注入法（ICSI）により作製した2細胞期胚の冷蔵輸送の検討

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超低温下（−196℃）における初期胚の凍結輸送に変わる手段として、より簡便な冷蔵下（4℃）における輸送方法について、高度な生産補助技術を必要とする卵細胞内精子注入法（ICSI）により作成された初期胚を用いて検討した。C57BL/6Jを用いた卵細胞内精子注入法（ICSI）後の卵子は52%/205/392が生存した。それらの受精率は73%/150/205であり、その後の2細胞期胚への発生率は83%/124/150であった。得られた2細胞期胚は少量のM2培地と共にテストチューブへ移し、冷蔵輸送は、クール宅急便にて近畿大学先端技術総合研究所（和歌山）から熊本大学・CARD（熊本）へ輸送した。輸送後の回収された生存胚は100%/122/122であった。さらに飼料飼育を講じたICR系マウスの卵管内に移植し発生能を検討したところ21%/26/122が産子へ発生した。以上の結果より、ICSI由来胚の冷蔵輸送が可能であることを明らかにした。

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