Development of a new device for manipulating frozen mouse 2-cell embryos on the International Space Station

Sayaka Wakayama (sayakaw@yamanashi.ac.jp)
University of Yamanashi
Mariko Soejima
University of Yamanashi
Yasuyuki Kikuchi
University of Yamanashi
Erika Hayashi
University of Yamanashi
Natsuki Ushigome
University of Yamanashi
Ayumi Hasegawa
RIKEN BioResource Research Center
Keiji Mochida
RIKEN BioResource Research Center
Tomomi Suzuki
Japan Aerospace Exploration Agency
Chiaki Yamazaki
Japan Aerospace Exploration Agency
Toru Shimazu
Japan Space Forum
Hiromi Sano
Japan Manned Space Systems Corporation
Masumi Umehara
Advanced Engineering Services Co., Ltd
Hitomi Matsunari
Meiji University
Atsuo Ogura
RIKEN BioResource Research Center
Hiroshi Nagashima
Meiji University
Teruhiko Wakayama

University of Yamanashi

Article

Keywords: Embryo, Vitrification, High osmolarity vitrification, Cryopreservation, International Space Station

Posted Date: April 4th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1508929/v1

Abstract

Whether mammalian embryos develop normally under microgravity remains to be determined. However, embryos are too small to be handled by inexperienced astronauts who orbit Earth on the International Space Station (ISS). Here we describe the development of a new device that allows astronauts to thaw and culture frozen mouse 2-cell embryos on the ISS without directly contacting the embryos. First, we developed several new devices using a hollow fiber tube that allows thawing embryo without practice and observations of embryonic development. However, the general vitrification method requires liquid nitrogen, which is not available on the ISS. Therefore, we developed another new device, Embryo Thawing and Culturing unit (ETC) employing a high osmolarity vitrification method, which preserves frozen embryos at −80°C for several months. Embryos flushed out of the ETC during thawing and washing were protected using a mesh sheet. Although embryonic development could not be observed in the ETC, thawed embryos formed blastocysts after 4 days of culture without direct contact. This ETC will enable untrained astronauts to thaw and culture frozen embryos on the ISS, as well as to serve as an important advance in fields such as clinical infertility and animal biotechnology.

Introduction

Sustaining life beyond Earth on space stations or on other planets will require a comprehensive understanding of how the characteristics of the environment in space, such as microgravity (mG) or space radiation, affect key phases of mammalian reproduction [1]. Performing experiments on the reproduction of mammals is difficult, and most studies on reproduction in space are therefore limited to nonmammalian species such as fish or amphibians [2-8]. However, the reproductive systems of the latter are fundamentally different from those of mammalian viviparity, which comprises pre- and post-implantation phases of embryonic development accompanies by the formation of the placenta. Therefore, the research outcomes of such studies cannot be extrapolated to advance our understanding of mammalian reproduction in space.

Previous studies conducted on the ISS compared with those on Earth show that ionizing radiation in space will not affect for the quality of mammalian spermatozoa for more than 200 years when they are preserved using freeze-drying [9,10]. However, it is not possible to generate mG for long periods on Earth, and the effects of mG on mammalian reproduction are therefore unknown. We previously considered the possibility of conducting research on mammalian embryonic development *in vitro* under simulated mG using a three-dimensional clinostat [11]. Embryo culture for 4 days under such conditions generates significant anomalies such as delayed embryonic development, deterioration of the trophectoderm, impaired rates of differentiation, and failure to develop viable offspring [12]. Similar results were published by other groups [13,14], suggesting that mG may adversely influence the development of preimplantation mammalian embryos. Thus, humans may not succeed in permanently colonizing other planets because of failed reproduction.

The experiments described above were simulations performed on the ground. Recently, Lei et al published a study of mouse embryos that were launched into orbit [15]. However, this experiment used live embryos, which developed by the time of launch and were unfortunately exposed to strong vibrations from rocket, thereby confounding the effects of mG on embryo development. To definitively study the effects of mG on embryonic development, frozen embryos must thawed and cultured on the ISS. However, because of the small sizes (80–100 mm) of mammalian oocytes and embryos, they are very difficult and sensitive to direct manipulation, and mouse embryos are culturable in vitro for only 4 to 5 days. Therefore it has not been possible to perform experiments by untrained personnel in not only orbit but also on the ground.

To overcome these formidable obstacles, we developed a simple system for thawing and culturing embryos, which will allow untrained personnel such as astronauts to perform embryo experiments on Earth and on the ISS.

Results

OptiCell^{TM} device

In the first attempt, we used a hollow fiber tube (HFT) [16] and an OptiCell^{TM} device. Mouse 2-cell embryos were inserted into the HFT, which was then inserted into a pipette tip attached to a 1-ml syringe and then immersed in LN_2 (Fig. 1A–D). The frozen embryos in the HFT were inserted into the OptiCell^{TM} immediately after thawing (Fig. 1E, F) and then washed and cultured through exchange with the rewarming solution and culture medium respectively. Those solutions were delivered using a 10-ml syringe (Fig. 1G) and then placed in an incubator (37°C, 5% CO_2) for 4 days. The HFT in the OptiCell^{TM} achieved 88% survival of thawed embryos.

Blastocytes eveloped from 81% of the 2-cell embryos after 4 days in culture, which closer to non-frozen control embryos (92%) (Table 1). Generally, thawing frozen embryos is strictly time-limited and requires intensive practice. However, this method could be performed without any practice, therefore it will be use in animal research facilities and infertility clinics. However, if the HFT was located on the inside edge of the OptiCell^{TM}, the embryos were difficult observe. Although hard shaking moved the HFT from edge to the center of the OptiCell^{TM}, this was difficult. Furthermore, the culture area of the OptiCell^{TM} (100 cm^2) was too large, making it difficult to find the HFT inside the OptiCell^{TM} using a microscope. The astronauts performing this experiment on the ISS must be able to easily to find the HFT inside the device, which requires limiting the culture area.

Mini-plate device

We next used a Mini-plate developed by the Japan Aerospace Exploration Agency (JAXA) instead of the OptiCell^{TM}. The structure of the Mini-plate is similar to that of the OptiCell^{TM}, but much smaller (Fig. 1H, I). When fresh embryos in the HFT were inserted into the Mini-plate as a control and cultured for 4 days in

the CO_2 incubator, 98% of the embryos developed into blastocysts, demonstrating that the Mini-plate was not cytotoxic (Table 1). However, the HFT more often resided on the edge of this device and was difficult to move to the center, even after vigorous shaking. Furthermore, the port of Mini-plate is too tight, requiring the use of a metal needle instead of pipette tip to insert the HTF (Fig. 1J). However, when the HTF was frozen inside the metal needle, the HFT cracked after thawing, and all embryos were lost during washing. To avoid cracking the HFT in the needle, the HFT was inserted into the Mini-plate before immersion in the vitrification solution. However, we were unable to perform vitrification within the 2-min limit, and none of the thawed embryos survived.

Cell Experimental Unit (CEU)

We next used a Cell Experimental Unit (CEU) developed by JAXA in place of the Mini-plate (Fig. 1K). The CEU can be assembled so that the HFT harboring embryos can be fixed to its center. When non-frozen embryos in the HFT were cultured in the CEU, 95% developed into blastocysts after 4 days of culture in the CO_2 incubator (Table 1), indicating that the CEU was not toxic to the embryos. However, because of the complex structure of the CEU (Fig. 1L), the HFT containing frozen embryos melted before CEU assembly was completed, even on dry ice. We attempted to place the HFT containing embryos into the vitrification solution onto the plate of CEU before freezing and then assembly, after which the CEU was immersed in LN_2. Unfortunately, we were unable to complete the process within the time limit, despite practice. We therefore abandoned the use of the CEU.

New Cell Experimental Unit (NCEU)

Next, we designed a new device based on the CEU, which we called the NCEU (Fig. 1M). The NCEU, which tolerates freezing in LN_2, is equipped with a silicone flipper that can pinch the HFT onto the center of plate (Fig. 1N). The culture area of the NCEU was easily covered by a gas-permeable membrane, which was attached using a nontoxic adhesive. When the HFT with fresh embryos was pinched onto the center of the NCEU and the medium was changed three times (Fig. 1O), the HFT could not be flushed out, and 100% of the embryos developed to blastocysts (Fig. 1P, Table 1) after 4 days of culture in the CO_2 incubator. When OptiCell^{TM} method was used, sometimes HFT was located on the inside edge and became difficult to observe embryos. However, this method allows the HFT to be fixed in the center and at the bottom of plate, making it easy to observe all embryos. Thus, we concluded that the NCEU was possibly the best device for thawing, washing, and observing embryos and that the procedures could be successfully performed without practice.

Restriction of freezer on ISS

Even if the frozen embryos are transported by rocket to the ISS, frozen embryos must be stored on the ISS for about a month before start experiment because the astronauts are too busy immediately after the

rocket's arrival. During the development of these devices, we were informed that liquid nitrogen was not available on the ISS. When using general vitrification method, the -95°C freezer at ISS cannot preserve frozen embryos, even for short periods of time. Therefore, we decided to use the high osmolarity vitrification (HOV) method, which allows storage of the embryos at −80°C for >1 month [17]. However, because the pore size of the membrane of the HFT was very small (5–15 nm), the vitrification solution (EFS42.5) used in the HOV method is highly viscous and cannot be exchanged with the thawing solution (0.75 M sucrose-PB1) through the HFT membrane within time limit. Therefore, we decided to start developing a new method that does not use HFT.

Frozebag® using a mesh bag

To achieve exchange of the viscous solution without flushing out the embryos, we used a mesh sheet (Fig. 2A), which was converted into a bag using a heat sealer (hereafter “mesh bag”) (Fig. 2B). A Frozebag® (Fig. 2C) served as the embryo culture device. First, to exam toxicity, we directly inserted fresh embryos into the Frozebag®, filled it with culture (CZB) medium, and the heat-sealed it. However, none of the embryos developed to the blastocysts, likely because of the bag's impermeability to gas (Table 2). Therefore, we developed a new embryo culture system, in which CZB medium was equilibrated with 5% CO_2 in a CO_2 incubator before use (hereinafter referred to as eCZB medium) (Figure 2D) [18]. Next, fresh embryos were directly placed in the Frozebag®, or inserted into the mesh bag on the scoop (Fig. 2E), placed in the Frozebag®, and then cultured for 4 days in eCZB medium (Fig. 2F). Under these conditions, 86-94% of embryos developed into blastocysts (Table 2), which demonstrated that the Frozebag®, the mesh bag, and the scoop were not cytotoxic.

Next, the mesh bag on the scoop was filled with EFS42.5, and 2-cell embryos were inserted into the mesh bag, frozen in LN_2, and the scoop then was placed in the Frozebag®, heat-sealed, and stored at −80°C for a few days. When the Frozebag® containing frozen embryos was thawed, washed two times via a port using syringes (Fig. 2G), and then cultured for 4 days, 50% of the embryos were recovered from the mesh bag, and none formed blastocysts. Furthermore, no embryo reached the blastocyst stage even when embryos were immediately harvested from the Frozebag® after thawing and cultured on a Petri dish for 4 days (37°C, 5% CO_2) (Table 2). This outcome may be explained by the possibility that the Frozebag® method failed to allow exchange of the vitrification solution with the dilution solution within the restricted time limit. Furthermore, the Frozebag® contained a 10-fold greater volume of EFS42.5 solution compared with the HOV method.

Frozebag® using a mesh cap

To avoid introducing a large volume of EFS42.5 solution into the Frozebag, we attempted to freeze the embryos directly on the inner surface of the Frozebag® in the presence of a small amount of EFS42.5 solution. To prevent flushing out the embryos during solution exchange, the mesh sheet was converted to

a cap shape (hereafter “mesh cap”) and attached to the inner port of the Frozebag® (Fig. 3A). When fresh embryos were inserted into the Frozebag®, the medium was exchanged twice. After 4 days of culture, 15 of 60 embryos were collected, among which 80% developed into blastocysts (Table 3). When embryos were frozen on the surface of the Frozebag®, thawed, washed, and cultured for 4 days, 23% were recovered and none survived (Table 3).

Frozebag® equipped with a cryotube and mesh wall

We assumed that the HOV method could be performed in a cryotube [17]. The material and thickness of the Frozebag® are completely different from those of the cryotube, and the difference in thermal conductivity during freezing may have damaged the embryos. Therefore, we used cryotubes to freeze the embryos and placed them in a Frozebag®, which was stored at −80°C for several days. The embryo recovery rate using a mesh cap from the Frozebag® ranged from 23% to 25%. We after testing several other methods, we decided to use a heat sealer to attach a mesh sheet to the middle of the Frozebag® to serve as a barrier (hereafter “mesh wall”) to prevent flushing out the embryos (Fig. 3B). When fresh embryos were inserted into cryotubes, which were placed in this modified Frozebag® with mesh wall and cultured for 4 days, 80% of the embryos were recovered. Blastocytes were produced by 91% of these embryos (Table 3).

Next, embryos were frozen in cryotubes, placed in a Frozebag®, and stored at −80°C for several days. When the Frozebag® was thawed, washed by exchanging the solutions, and cultured for 4 days in eCZB medium, we obtained two blastocysts (2%). Although the morphological quality of those blastocysts was quite poor (Fig. 3C), this was the first successful attempt to culture frozen embryos to produce blastocytes without directly manipulating the embryos.

The low yield of blastocysts may be explained by insufficient removal of the EFS42.5 solution from the Frozebag®, even after multiple exchanges of the solution. Next, we opened the Frozebag® immediately after thawing, collected the embryos, and cultured them on a Petri dish in the CO_2 incubator. This procedure yielded 48 of 50 embryos, among which 16 (55%) developed into blastocysts (Table 3). Hereafter, we called this modified Frozebag® as an Embryo Thawing and Culturing unit (ETC).

ETC using V-tube

To improve the efficiency of exchanging the EFS42.5 solution with eCZB medium, the size of the cryotubes was reduced to the extent possible (Fig. 3D). We called the smallest cryotube a “V-tube.” We simultaneously attempted to increase the number of medium exchanges to completely remove residual vitrification/dilution solution from ETC (Fig. 3E). Embryos were frozen using the V-tube, placed in the ETC, stored at −80°C for a few days, thawed with an additional medium exchange 1-h later, and cultured for 4 days. We recovered 61 of 98 embryos (62%), and 30 (59%) developed into good-quality blastocysts (Fig. 3F, Table 3). We succeeded in obtaining good-quality blastocysts, as described in the next paragraph,

when one or two additional medium exchanges were performed 2-h later or 30 min and 2-h later, although the rate of survived embryo decreased to15%–20%.

To evaluate the quality of the embryos obtained using this system, morulae/blastocysts cultured for 3 days in ETC were transferred to mice. These embryos yielded 21 (31%) offspring (Fig. 3G, Table 4), which is same rate achieved when embryos cultured in vitro were transferred in my laboratory [19]. This result shows that the ETC generated normal morulae/blastocysts.

Preservation period of embryos at −80°C and reproducibility of this method

When the μG experiment is conducted on the ISS, storage in the −95°C freezer may exceed 1 month. Therefore, we determined our ability to preserve frozen embryos at −80°C in the ETC. When the ETC was stored for 1 month at −80°C, 17 of 52 (32.7%) embryos developed into blastocysts. The blastocyst generation rate decreased with longer storage, although some embryos developed into blastocysts after storage for 3 months (Table 5). Furthermore, we determined whether inexperienced people were able to obtain blastocysts using the ETC. For this purpose we used 2 ETCs with 180 vitrified embryos. Two people thawed and cultured the embryos. Although the rate of blastocyst generation was not high, both person obtained good blastocysts (Table 5).

Discussion

Here we describe the development and validation of the ETC, which thaws, washes, and cultures frozen mouse 2-cell embryos without direct contact. We obtained good-quality blastocysts using this method, which was judged by their ability to develop into healthy offspring. Moreover, such embryos were produced using the ETC after storage for at least for 1 month at −80°C, suggesting that the ETC will serve as an ideal device for experiments conducted in Earth orbit. However, such experiments are expensive and possibly difficult to repeat. We therefore must optimize the recovery and developmental rate of embryos after thawing and culturing in the ETC.

For this purpose, we developed equilibration medium that allowed us to culture embryos in a closed gas-impermeable container such as the Frozebag® [18], and we identified the most appropriate stage of mouse 2-cell embryos for the present project [19]. Moreover, to safely transport the experimental materials to the ISS, the experimental equipment must be sterilized using high-dose gamma sterilization. Therefore, the solutions and ETC must not be altered by gamma-ray sterilization. Furthermore, the vibration of the rocket during launch and the forces encountered during the landing of the recovery vehicle on Earth may damage the experimental materials. Moreover, approximately 1 month is required to recover the ETC from the ISS subsequent to completing the experiment, and the embryos must be fixed with PFA and stored at 4°C during this time. We therefore must establish techniques to analyze fixed embryos, such as immunostaining, next-generation sequencing, or both.

Although the ETC achieves a high developmental rate of thawed 2-cell embryos, we were unable to recover all embryos after exchanging several solutions and media. We must therefore determine where the embryos are flushed out from Frozebag® and improve the ETC to achieve a 100% embryo recovery rate. Furthermore, the optimal time for medium exchange must be determined. These variables must by optimized to ensure the success of experiments conducted on the ISS. On the other hand, although devices such as the NECU are not suitable for use on the ISS, on the ground, the recovery rate of blastocysts is very high (Table 1). Thus, these methods do not require skillful handling of embryos, enabling the use of these devices in orbit as well as in animal research facilities and infertility clinics.

Methods

Mice

Female and male BDF1 mice were obtained from SLC Inc. (Hamamatsu, Japan). ICR mice were bred in our mouse facility. Embryos aged 8–10 weeks were implanted into pseudopregnant ICR mice that were mated with vasectomized ICR males. On the day of the experiment, or upon completion of all experiments, the mice were killed using CO_2 inhalation or cervical dislocation. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of the Laboratory of Animal Experimentation of the University of Yamanashi (reference number: A29-24), which followed the ARRIVE guidelines.

Media

HEPES-CZB (H-CZB) medium [20] was used to collect embryos from oviducts, and CZB medium [21] was used to culture embryos in a humidified incubator (5% CO_2, 37°C).-Vitrification solution for HFT method: Equilibration and vitrification solutions were used for vitrification, and the rewarming, dilution, and washing solution were used for thawing [16]. The vitrification solutions for the HOV method included EFS20 and EFS42.5. The embryos were thawed using 0.25 M and 0.75 M sucrose-PB1 (S-PB1) [17]. Equilibrated CZB (eCZB) medium was prepared as previously described [18]. Briefly, CZB medium was used to fill the container, the lid was placed slightly off-center, and the container was then placed in the CO_2 incubator for >24 h to equilibrate the CO_2 concentration of the medium with that of the atmosphere. The eCZB medium was prepared 1 day before commencing the experiment.

In vivo fertilization

Female mice were induced to superovulate by injection of 5 international units (IU) of equine chorionic gonadotropin (eCG) followed 48-h later by injection of 5 IU of human chorionic gonadotropin (hCG). These female mice were immediately mated and examined the following day for vaginal plugs, after which they were separated from the males. The next day, 2-cell embryos were recovered by flushing the

oviducts with H-CZB medium and incubating then in CZB medium at 37°C in an atmosphere containing 5% CO_2.

Preparation of HFTs

HFTs are straw-like structures with a reticulated membrane that tolerate freezing at −196°C and are nontoxic for mammalian embryos. HFTs are used to simplify the vitrification of embryos [16]. We reasoned that if embryos were frozen inside an HFT, the astronauts could thaw and culture the embryos without directly contacting them. The internal diameter of an HFT (185 μm) and the pore sizes of the membrane range between 5–15 nm, which allows exchange solutions through HFT membrane but not lose any embryos (Fig. 1A). The HFT comprises a transparent film, which allows visual observations of embryos morphology during the 4-day culture.

HFT vitrification

The vitrification of 2-cell embryos using the HFT was performed using a modified published method [16]. Briefly, approximately 10 2-cell embryos were placed in the equilibration solution and then aspirated into the HFT (Fig. 1A), which was sealed by pinching its ends using a tweezers. Following equilibration (5–7 min), the HFT containing embryos was transferred to the vitrification solution placed on an ice bath. Next, the embryos were aspirated into a 1-ml syringe attached with a small pipette tip (for 20-μl use) (Fig. 1B, C), and the syringe was immersed in LN_2 within 1–2 min (Fig. 1D).

OptiCell™ method

OptiCell™ (Thermo Fisher Scientific) is a commercially available cell culture device, which comprises a transparent CO_2-permeable membrane and two ports for medium exchange. For thawing, the syringe was removed from the LN_2 (Fig. 1E) and then the pipette tip attached to the syringe was inserted into a port of the OptiCell™, previously filled with thawing solution. Within 1 min, the HFT with embryos was ejected from the syringe into the warm OptiCell™ (Fig. 1F). Next, a 10-ml syringe containing dilution solution and an empty 10-ml syringe were attached to both ports of the OptiCell™. Within 2 min, dilution solution was injected into the OptiCell™, and the thawing solution was removed using the empty syringe (Fig. 1G). After 3 min, the dilution solution was similarly replaced with washing solution, after 5 min, the washing solution was replaced with CZB medium, and then the OptiCell™ with the HFT was placed in a CO_2 incubator for 4 days.

Mini-plate method

Mini-plates comprise a transparent CO_2-permeable membrane and two ports as in the OptiCell ™, but are much smaller (3 cm × 2 cm) than those of the OptiCell ™ (Fig. 1H). Experiments using a Mini-plate were conducted under the same conditions used for the OptiCell ™, with the exception that a 21-gage needle was used to insert the HFT into the Mini-plate, because the inner diameter of port was too small and tight. In other experiments, the vitrification solution was added to the Mini-plate, and the equilibrated embryos in the HTF were the injected into the Mini-plate, which was placed in the vitrification solution. Within 1 min, the Mini-plate was immersed in LN_2.

Cell Experimental Unit (CEU) method

Embryos in the HFT were prepared using the OptiCell method, except that the HFT with frozen embryos was placed on the center of the window of the disassembled CEU, assembled within time limit, and stored in LN_2. In some experiments, vitrification solution was placed on the windows of the disassembled CEU, the equilibrated embryos in the HTF were placed into the vitrification solution, and then the CEU was assembled and immersed in LN_2.

New Cell Experimental Unit (NCEU) method

To serve as a control, the HFT with fresh embryos was pinched onto the center of the NCEU using a silicone flipper (Fig. 1N), and a gas-permeable membrane, which was used to cover the culturing area of the NCEU, was fixed using a nontoxic adhesive. After medium exchange, the NCEU was placed in an incubator (5% CO_2, 37°C) for 4 days.

Frozebag® with a mesh bag

The Frozebag® (NIPRO Co. Japan), which provides two ports for medium exchange, is easily processed using a heat sealer, withstands LN_2, but is not gas-permeable (Fig. 2C). The pore size of the mesh sheet (NBC meshtec Inc, Japan) is approximately 20 μm (Fig. 2B), and the embryos cannot be observed during the experiment. However, in the absence of an alternative, we abandoned to observe embryos on the ISS. We used a heat sealer to convert the sheet into a 1 cm × 2-cm bag (hereafter “mesh bag”) (Fig. 2B).

To serve as a suitable control experiment, the mesh bag was placed on the scoop, which was used as a saucer (NIPRO Cell Sleeper, Japan), and filled with eCZB medium (Fig. 2E). Next, 10–20 fresh embryos were inserted into the mesh bag through a small slit. The scoop with the mesh bag was placed in a Frozebag®, which was heat-sealed and filled with eCZB medium through the port (Fig. 2G). The Frozebag® was then placed in an incubator (5% CO_2, 37°C) for 4 days. For experiment, embryos were transferred to the vitrification solution (EFS20) on the Petri dish and incubated for 2 min at room temperature. Next, the embryos were transferred into vitrification solution (EFS42.5) on the Petri dish, and within 1 min, were transferred into the mesh bag, which was filled with EFS42.5 on the scoop. The scoop

was then immersed in LN_2. After vitrification, the scoop was inserted into the Frozebag® in LN_2, which was then removed and immediately heat-sealed, after which it was returned to LN_2 and stored at −80 ºC.

Thawing and culturing embryos using a Frozebag®

For thawing embryos, the Frozebag® was removed from the freezer and injected with the first dilution solution (0.75 M S-PB1) through a 30-ml syringe via the port within 1.5 min. After 8.5 min, this solution was exchanged with the second dilution solution (0.25 M S-PB1) and the waste solution was simultaneously drained from another port. After 3 min, this solution was exchanged with the culture medium (eCZB). Finally, the medium was similarly exchanged with eCZB medium at one hour later and placed in an incubator (5% CO_2, 37°C) for 4 days.

Collection of embryos from the Frozebag®

Upon completion of the culture period, the cut the end of the Frozebag® and the mesh bag with medium were transferred to a 10-cm dish. The mesh bag was opened using tweezers and gently shaken to release the embryos. The embryos were observed using a microscope to determine the recovery and developmental rates. In some experiments, the embryos were further cultured on a dish in an incubator (5% CO_2, 37°C).

Frozebag® with a mesh cap

To prevent the loss of embryos during solution exchange, a mesh sheet cap was attached to the inner port of the Frozebag® (Fig. 3A). To serve as a control, fresh embryos were placed on the surface of a Frozebag®, filled with eCZB medium, and placed in an incubator (5% CO_2, 37°C) for 4 days. Embryos were transferred into a 50 µl-drop of vitrification solution (EFS 20) on the Perti dish for 2 min, transferred into a 50 µl-drop of vitrification solution (FEF42.5) on the inside surface of the Frozebag®, which was heat-sealed within 1 min. Next, the Frozebag® was immediately frozen in LN_2 and stored at −80°C. The embryos were thawed as described above.

Frozebag® with a cryotube or a V-tube

To prevent the loss of embryos during solution exchange, a mesh sheet wall was attached to the middle of the Frozebag® (Fig. 3B). Since the Frozebag® and mesh sheet do not stick together by heat-sealed, a piece of plastic bag (Ziploc, Asahi KASEI, Japan) was placed in the gap between Frozebag® and mesh sheet, which was used as an adhesive to heat seal the bags, making them adhere perfectly. For vitrification, 10–30 embryos were suspended in EFS20 equilibrium solution for 2 min and then transferred into a cryotube (Sumitomo Bakelite, Japan) or V-tube, fabricated in-house, (Fig. 3D) containing 50 µL of

EFS42.5. After 1 min, the cryotube or V-tube was plunged directly into LN_2. These tubes were then placed in the Frozebag® under LN_2, heat-sealed as described above, and then stored at −80 ºC. Thawing was performed as described above.

Embryo transfer

Embryos (2-cell stage) collected from the Frozebag® just after thawing, morulae/blastocysts cultured for 3 days, or blastocysts cultured for 4 days were transferred to the oviduct of day 0.5 or uteri of day 2.5 pseudopregnant mice mated with vasectomized males [22]. Embryos (n = 5–8) were transferred into each oviduct. On day 18.5, the offspring were delivered by cesarean section and allowed to mature.

Statistical analysis

Survival, developmental, and birth rates were evaluated using chi-squared tests. Statistically significant differences between the variables are defined as $p < 0.05$.

Declarations

Acknowledgements

We thank Dr. A. Higashibata, Dr. T. Yamamori, Dr T. Kohda, Dr S. Kishigami, Dr M. Ooga, Dr Y. Fujimoto, Mrs. I. Osada, C. Yamaguchi, and Y. Kanda for assistance in preparing this manuscript. This work was partially funded by the Naito Foundation (S.W) and Takahashi Industrial and Economic Research Foundation (189) to S.W.; Asada Science Foundation (T.W) and the Canon Foundation (M20-0006) to T.W. The authors would like to thank Enago for the English language review.

Author contributions

S.W. and T.W. conceived and designed the study. S.W., S.M., K.Y., H.E., U.N., H.A., M.K., S.T., Y.C., S.T., U.M., S.H., M.H., O.A., N.H., and T.W. performed experiments, analyzed the data, and interpreted the results. S.W. and T.W. wrote the manuscript. All authors read and edited the manuscript.

Declaration of competing interests

The authors declare that they have no competing interests.

Availability of Data and Materials

All data generated or analysed during this study are included in this published article

References

1. Mishra, B. & Luderer, U. Reproductive hazards of space travel in women and men. *Nat Rev Endocrinol* **15**, 713-730, doi:10.1038/s41574-019-0267-6 (2019).
2. Aimar, C.*et al.* Microgravity and hypergravity effects on fertilization of the salamander Pleurodeles waltl (urodele amphibian). *Biol Reprod* **63**, 551-558 (2000).
3. Ijiri, K. Ten years after medaka fish mated and laid eggs in space and further preparation for the life-cycle experiment on ISS. *Biological sciences in space = Uchu seibutsu kagaku* **18**, 138-139 (2004).
4. Schatten, H.*et al.* Effects of spaceflight conditions on fertilization and embryogenesis in the sea urchin Lytechinus pictus. *Cell biology international* **23**, 407-415 (1999).
5. Serova, L. V. [Effect of weightlessness on the reproductive system of mammals]. *Kosmicheskaia biologiia i aviakosmicheskaia meditsina* **23**, 11-16 (1989).
6. Souza, K. A., Black, S. D. & Wassersug, R. J. Amphibian development in the virtual absence of gravity. *Proc Natl Acad Sci U S A* **92**, 1975-1978 (1995).
7. Tash, J. S., Kim, S., Schuber, M., Seibt, D. & Kinsey, W. H. Fertilization of sea urchin eggs and sperm motility are negatively impacted under low hypergravitational forces significant to space flight. *Biol Reprod* **65**, 1224-1231 (2001).
8. Ubbels, G. A., Berendsen, W. & Narraway, J. Fertilization of frog eggs on a Sounding Rocket in space. *Adv Space Res* **9**, 187-197 (1989).
9. Wakayama, S.*et al.* Evaluating the long-term effect of space radiation on the reproductive normality of mammalian sperm preserved on the International Space Station. *Sci Adv* **7**, doi:10.1126/sciadv.abg5554 (2021).
10. Wakayama, S.*et al.* Healthy offspring from freeze-dried mouse spermatozoa held on the International Space Station for 9 months. *Proc Natl Acad Sci U S A* **114**, 5988-5993, doi:10.1073/pnas.1701425114 (2017).
11. Tremor, J. W. & Souza, K. A. The influence of clinostat rotation on the fertilized amphibian egg. *Space life sciences* **3**, 179-191 (1972).
12. Wakayama, S.*et al.* Detrimental effects of microgravity on mouse preimplantation development in vitro. *PLoS One* **4**, e6753, doi:10.1371/journal.pone.0006753 (2009).
13. Kojima, Y.*et al.* Effects of simulated microgravity on mammalian fertilization and preimplantation embryonic development in vitro. *Fertil Steril* **74**, 1142-1147, doi:10.1016/s0015-0282(00)01583-1 (2000).
14. Wang, Y.*et al.* A major effect of simulated microgravity on several stages of preimplantation mouse development is lethality associated with elevated phosphorylated SAPK/JNK. *Reprod Sci* **16**, 947-959, doi:10.1177/1933719109337544 (2009).
15. Lei, X. H.*et al.* Development of mouse preimplantation embryos in space. *Natl Sci Rev* **7**, 1437-1446, doi:10.1093/nsr/nwaa062 (2020).
16. Matsunari, H.*et al.* Hollow fiber vitrification: a novel method for vitrifying multiple embryos in a single device. *J Reprod Dev* **58**, 599-608 (2012).

17. Mochida, K.*et al.* High osmolality vitrification: a new method for the simple and temperature-permissive cryopreservation of mouse embryos. *PLoS One* **8**, e49316, doi:10.1371/journal.pone.0049316 (2013).
18. Kikuchi, Y., Wakayama, S., Ito, D., Ooga, M. & Wakayama, T. Optimised CO2-containing medium for in vitro culture and transportation of mouse preimplantation embryos without CO2 incubator. *PLoS One* **16**, e0260645, doi:10.1371/journal.pone.0260645 (2021).
19. Hayashi, E.*et al.* Mouse in vivo-derived late 2-cell embryos have higher developmental competence after high osmolality vitrification and -80 degrees C preservation than IVF or ICSI embryos. *J Reprod Dev*, doi:10.1262/jrd.2021-115 (2022).
20. Kimura, Y. & Yanagimachi, R. Intracytoplasmic sperm injection in the mouse. *Biol Reprod* **52**, 709-720 (1995).
21. Chatot, C. L., Lewis, J. L., Torres, I. & Ziomek, C. A. Development of 1-cell embryos from different strains of mice in CZB medium. *Biol Reprod* **42**, 432-440 (1990).
22. Inoue, R., Harada, K., Wakayama, S., Ooga, M. & Wakayama, T. Improvement of a twice collection method of mouse oocytes by surgical operation. *J Reprod Dev* **66**, 427-433, doi:10.1262/jrd.2020-059 (2020).

Tables

Tables 1 to 5 are available in the Supplementary Files section

Figures

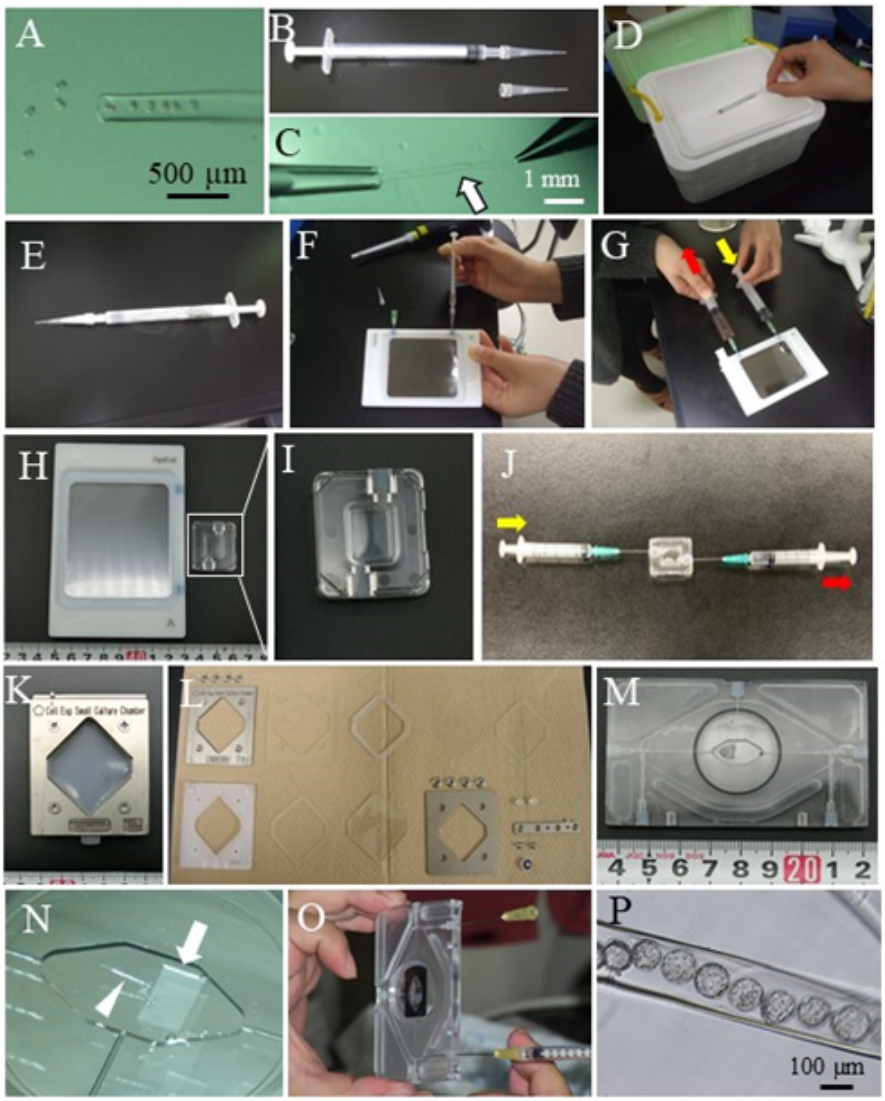

Figure 1. Vitrification devices with hollow fiber tube. (A) Mouse 2-cell embryos were aspirated into the HFT. (B) Pipette tip attached with syringe. (C) An HFT with embryos was inserted into a pipette tip. Arrow indicates the HFT with embryos. (D) The syringe was immersed into LN_2. (E) Immediately after removal from LN_2. (F) The HFT inside the pipette tip was inserted into the port of an OptiCell™. (G) Solution/medium exchange using a 10-ml syringe via the port. (H) OptiCell™ (left) and Mini-plate (right). (I) High magnification of the Mini-Plate. (J) Solution/medium exchange via the port. (K) Cell Experimental Unit (CEU). (L) Disassembled CEU. (M) New Cell Experimental Unit (NCEU). (N) The NCEU was equipped with a silicone flipper (arrow) used to pinch the HFT (arrowhead) onto the center of the plate. (O) Solution/medium exchange. (P) Blastocysts inside the HFT. Embryos were harvested from the NCEU after 4 days.

Figure 1

Please See image above for figure legend.

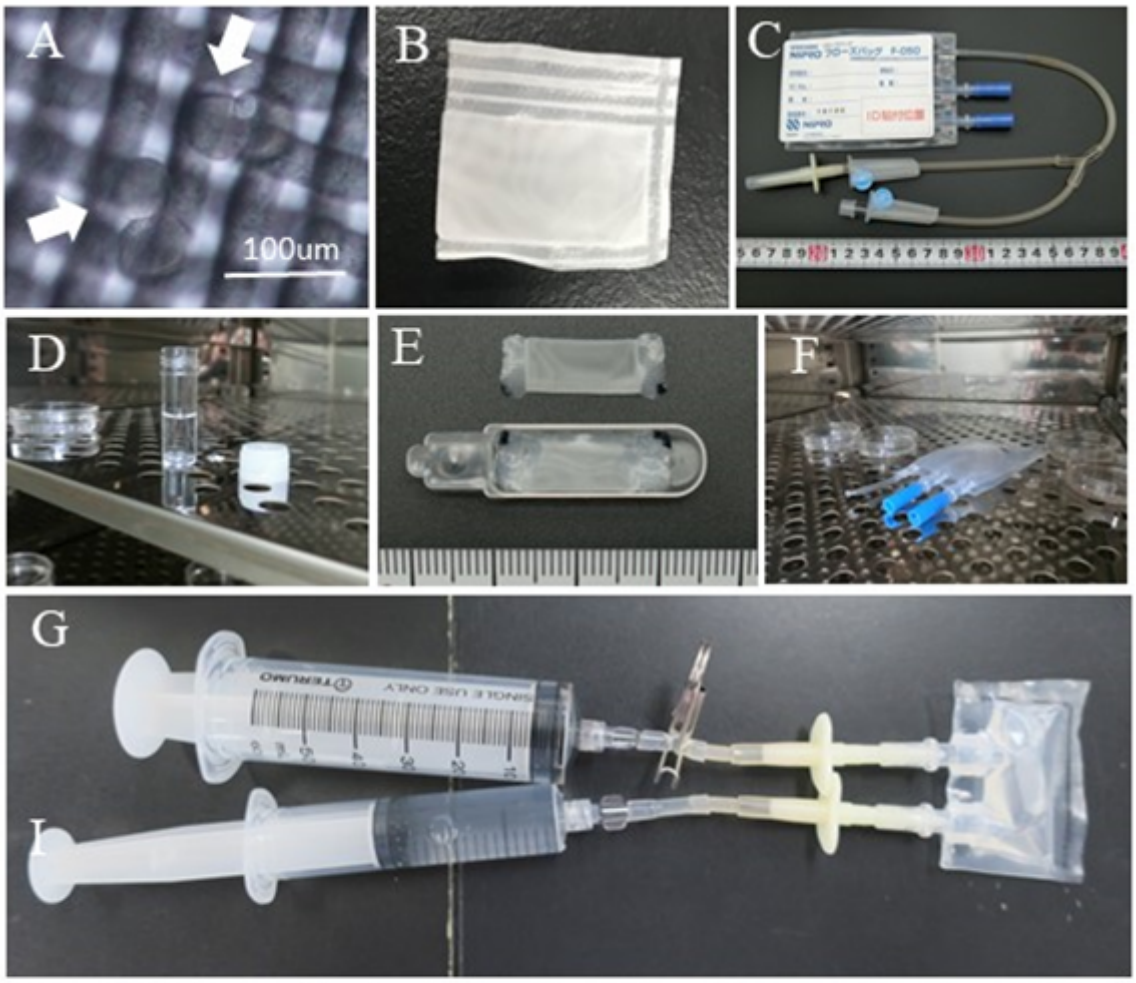

Figure 2. High osmolarity vitrification using a Frozebag® and mesh sheet. (A) Mesh sheet and embryos (arrows). (B) Mesh bag. (C) Frozebag® before modification. (D) Equilibration with CZB medium in an incubator (5% CO_2, 37° C). (E) Mesh bag on the scoop. (F) Incubation of the Frozebag® (5% CO_2, 37° C). (G) Solution/medium exchange using 30 ml and 50-ml syringes.

Figure 2

Please See image above for figure legend.

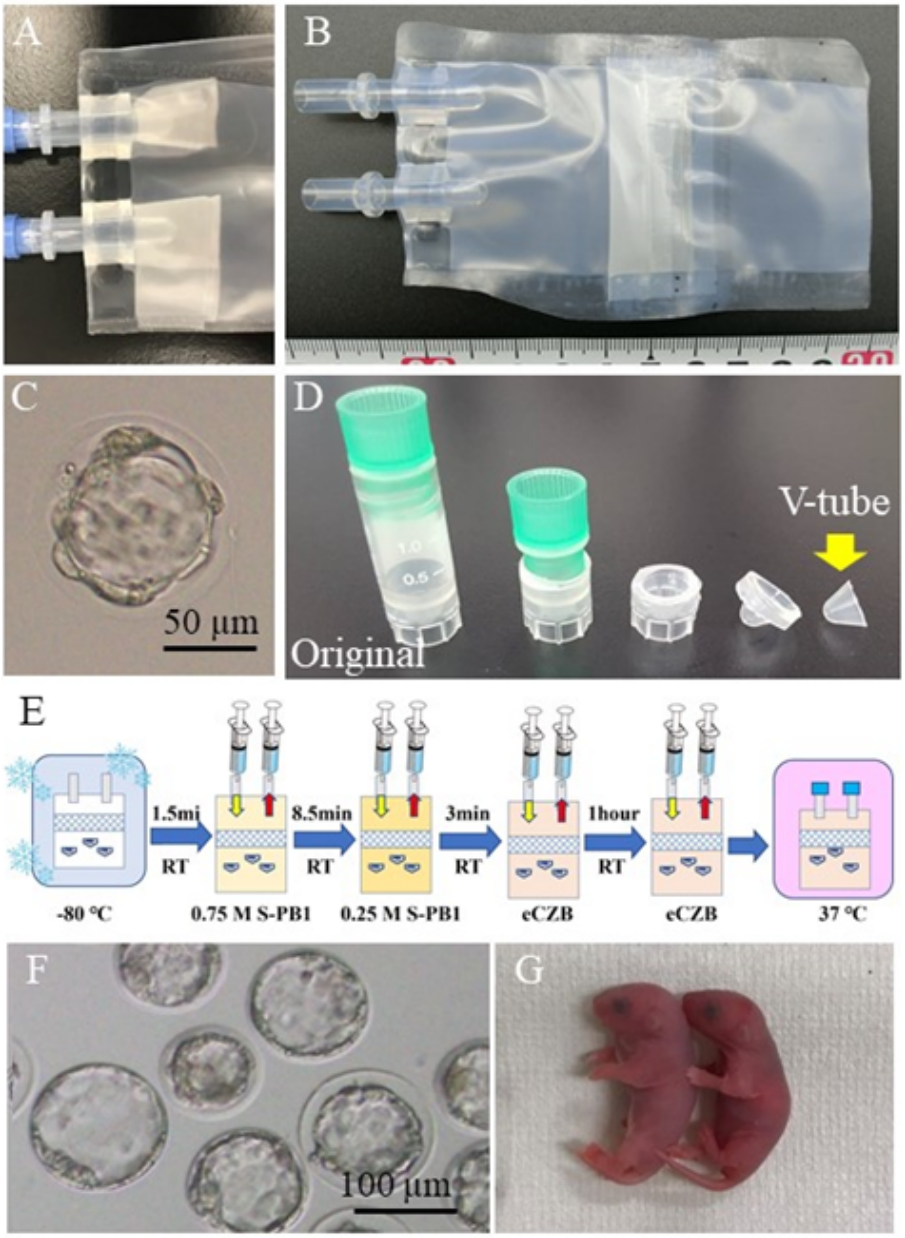

Figure 3. Embryo Thawing and Culturing unit (ETC). (A) Frozebag® with mesh cap. (B) Frozebag® with mesh wall. (C) Blastocyst collected from the Frozebag®. Although the quality of this embryos was insufficient, this was the first time that an embryo was thawed, washed, and cultured for 4 days without direct contact. (D) The top of cryotube was cut to produce the smallest tube (V-tube). (E) Protocol for thawing, washing, and culturing embryos in the ETC. (F) Blastocysts were harvested from the ETC after culture for 4 days. (G) Healthy offspring developed from the morulae/blastocysts derived from 3 days cultured embryos in ETC.

Figure 3

Please See image above for figure legend.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.png
- Table2.png
- Table3.png
- Table4.png
- Table5.png