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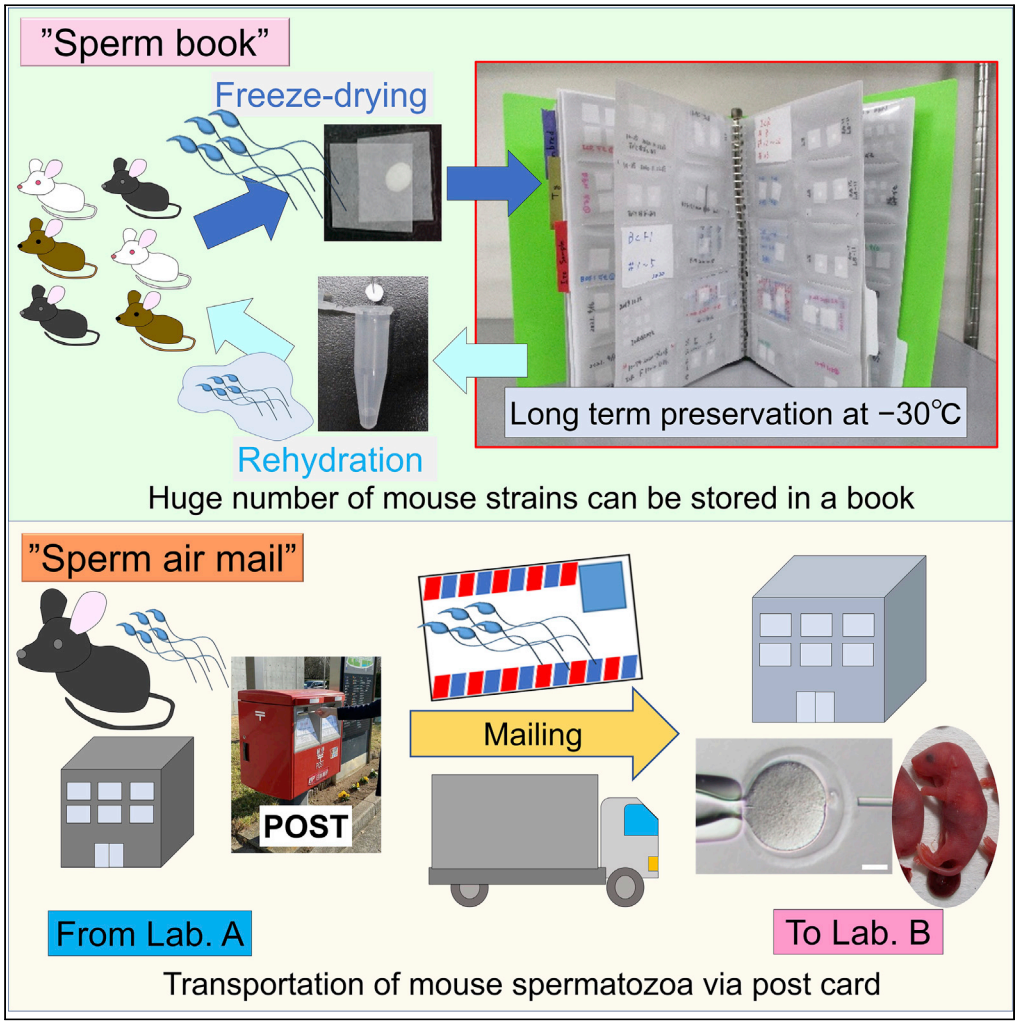
Mailing viable mouse freeze-dried spermatozoa on postcards



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Highlights

Successful preservation of mouse freeze-dried (FD) spermatozoa by thin plastic sheet

DNA integrity and developmental potential are comparable to the conventional method

Viable mouse FD sperm can be transferred via an air mail without any protection

Tens of thousands of mouse strains can be preserved in a single "sperm-book"

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Mailing viable mouse freeze-dried spermatozoa on postcards

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SUMMARY

Freeze-drying techniques allow the preservation of mammalian spermatozoa without using liquid nitrogen. However, the current method requires the use of glass ampoules, which are breakable, expensive, and bulky to store or transport. In this study, we evaluated whether mouse freeze-dried (FD) spermatozoa can be preserved and transported on thin materials. In this study, we demonstrated that FD sperm can be preserved in thin plastic sheets. Its DNA integrity was comparable to that of glass ampoule spermatozoa, and healthy offspring were obtained after preservation at −30°C for more than 3 months. We attached preserved FD sperm to postcards, and transported these to other laboratory inexpensively at room temperatures without any protection. This method will facilitate the preservation of thousands of mouse strains in a single card holder, promote collaboration between laboratories, conservation of genetic resources, and assisted reproductive technology.

INTRODUCTION

Preservation of mammalian spermatozoa plays an important role in infertility treatments, livestock production, maintenance of strains of genetically modified individuals, and conservation of genetic resources, including those of endangered species. Typically, spermatozoa are cryopreserved in liquid nitrogen (Benson et al., 2012), and healthy offspring are obtained through artificial insemination or *in vitro* fertilization after thawing (Sztein et al., 2018). However, the use of liquid nitrogen has several drawbacks. An ultra-low temperature of −196°C means that it must be handled with care, as there is a risk of suffocation that might be caused by saturation of nitrogen gas in the storage room, and it may not be readily available in some countries and regions. Most importantly, maintenance costs are expensive due to the constant need for re-filling liquid nitrogen, which makes it difficult for some countries to maintain their genetic resources. It is possible to preserve spermatozoa in a deep freezer at −150°C, but this is also expensive because it requires a continuous electric power supply. In addition, if power outages or disasters occur and sufficiently low temperatures are not maintained, storage cannot be continued.

To solve this problem, we developed a technique for mouse freeze-drying spermatozoa (Wakayama and Yanagimachi, 1998) and stored sperm at 4°C for 3 months, and at room temperature (RT) for 1 month in glass ampoules, without impairing the viability of the sperm. After freeze-drying, although all spermatozoa died, the DNA remained intact, and healthy offspring were obtained when spermatozoa were injected into oocytes. This technique has also been applied to the sperm of other species, such as rats, hamsters, rabbits, horses, and sheep (Choi et al., 2011; Gil et al., 2014; Hirabayashi et al., 2005; Liu et al., 2004; Muneto and Horiuchi, 2011; Palazzese et al., 2020). Recently, we found that the nuclei of mouse freeze-dried (FD) spermatozoa have a strong tolerance to frequent temperature changes compared to that of fresh spermatozoa (Wakayama et al., 2019). Moreover, when the vacuum condition of the glass ampoule was improved, healthy offspring were obtained from FD sperm stored at RT for more than a year without degrading their success rates (Kamada et al., 2018). As this technique does not require liquid nitrogen during preservation, mouse spermatozoa could be sent to the International Space Station, which has never been possible before, and the effects of space radiation on mammalian germ cells could be examined (Wakayama et al., 2017, 2021).

However, the storage of FD sperm in glass ampoules has the following problems: glass ampoules can be broken by falling due to shaking during earthquakes or through human error, which makes it impossible to continue storing sperm afterward. Glass ampoules are quite small, but take up a lot of space when

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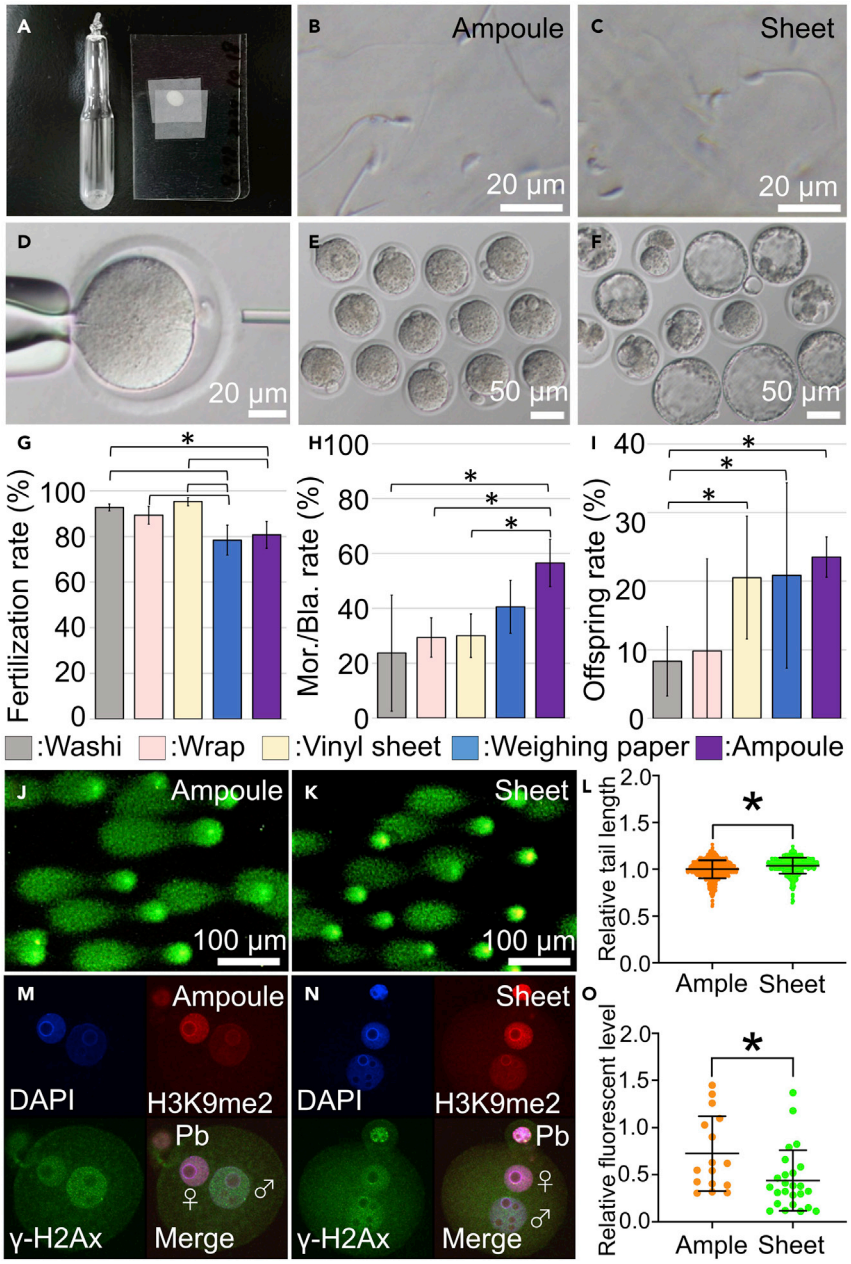

Figure 1. Evaluation of the quality of freeze-dried (FD) sperm preserved in a plastic sheet

(A) FD sperm were preserved in glass ampoules (left) or thin plastic sheets (right). Some sperm heads were separated from the tails after rehydration, but the rate of those sperm was no different between the ampoule (B) and plastic sheets (C). (D) Intracytoplasmic sperm injection (ICSI) using FD sperm derived from plastic sheet. Pronuclear formation (E) and blastocysts (F) were observed after ICSI. The rate of pronuclear formed zygote (G), developmental rate to the morula/blastocyst stage (H), and the rate of full-term development (I) after ICSI. Mor./Bla.: Morula and Blastocyst. Significance was determined by the chi-squared test, *: $p < 0.05$. Comet tail length of FD sperm was compared between glass ampoule (J) and plastic sheet (K), and the data was plotted (L). Orange dots represent the score of FD sperm derived from glass ampoules and green dots represent the score of plastic sheets. The length of comet tails was calculated relative to the mean length of FD sperm in glass ampoules. γ-H2Ax staining of male pronuclei derived from FD sperm was compared between glass ampoule (M) and plastic sheet (N), and the data was plotted (O). Images show DAPI-stained blue for male and female pronuclei (upper left), discrimination of female pronucleus by immunostaining for H3K9me2 with red (upper

Figure 1. *Continued*
right). Green foci staining with γ-H2Ax indicate DNA double-strand breaks (lower left) and merges (lower right), respectively. Orange dots and green dots indicate the brightness of the male pronuclei of spermatozoa in glass ampoule and plastic sheet, respectively. The comet assay and gamma-H2Ax assay were analyzed using the Wilcoxon-Mann-Whitney nonparametric test, *: $p < 0.05$.

thousands of ampoules are stored. Thus, it would be worth developing a more secure, space-saving, and simple method to store not only thousands of mouse strains but also to conserve genetic resources, especially of endangered species.

In this study, we attempted to store FD sperm in thin materials as an alternative to the glass ampoule method. If this method were found to be feasible, it would enable the safe management of a large number of mouse strains in a single book, such as a card folder, and also help to transport them internationally at a very low cost.

RESULTS

Weighing paper is the optimal material for preservation of spermatozoa

To find an optimal material as a vehicle for mouse FD spermatozoa, using Institute of cancer Research (ICR) strain male mouse, we first examined six different materials for estimating their ease of handling and efficacy of sperm collection: Washi (Traditional Japanese paper), Wrap, Vinyl sheet, Weighing paper, Filter paper, and Oblate. The characteristics and properties of those materials were mentioned in STAR Methods section, and used after processed into sheet-form (Figures 1A and S1). As shown in Table S1, Filter paper was judged as Unavailable, because the sperm drop stuck to the fibers of the Filter paper and could not be retrieved after freeze-drying. Oblate was also judged as Unavailable. Before start experiment, it was assumed that it would be easier to collect FD sperm from Oblate as it dissolves in water. However, there was a very large amount of undissolved residue, which caused difficulty in collecting spermatozoa. Other four materials could be used to collect spermatozoa after drying. However, Wrap was easy to tear and the Vinyl sheet easily lost the drop of frozen spermatozoa before the drying process, therefore those were judged as Hard or Difficult, respectively. Washi was relatively good material but there were a lot of contamination of materials after rehydration, and became difficult to collect FD sperm before ICSI. Weighing paper did not permit the sperm drop to fall before the drying process; in addition, it was thinner than the Vinyl sheet and tougher than Wrap. Thus, of the six materials, Weighing paper was the easiest to handle.

Next, spermatozoa were freeze-dried on four materials, i.e. Washi, Wrap, Vinyl sheet, and Weighing paper, put the material between thin plastic sheets (polyethyleneterephthalate) and sealed them without air as much as possible, and preserved for one month at −30°C. When intracytoplasmic sperm injection (ICSI) was performed using the FD sperm (Figures 1B–1D), the fertilization rates were comparable between all materials (Figures 1E and 1G); however, when developmental rates to morulae/blastocyst were examined, the highest rate was obtained in Weighing paper (Figures 1F and 1H; Table S2). As the ability of full-term development is the most important criteria for successful preservation, embryos derived from FD sperm preserved in plastic sheets were transferred into the oviduct at the two-cell stage, and the offspring rate was examined. As a result, many offspring were obtained from FD sperm preserved in the thin plastic sheets, and the highest offspring rate was obtained from FD sperm that was preserved using Weighing paper, which was comparable to those from the control (21% vs. 24%, respectively) (Figure 1I; Table 1). Thus, considering the ease of handling and the offspring rate, we judged that Weighing paper was the most suitable material for storage of FD sperm.

DNA integrity of FD sperm preserved by plastic sheet is comparable to that of conventional method

To evaluate in more detail the quality of FD spermatozoa stored in plastic sheet, DNA integrity of FD sperm preserved for 1 day at −30°C was compared with that of sperm preserved in a glass ampoule. First, we examined the length of the comet tails using the comet assay and calculated the relative values. The comet lengths of the spermatozoa from the glass ampoule were slightly but significantly shorter than those from the plastic sheet (Figures 1J–1L; Table S3). Second, we measured the brightness of the male pronucleus after ICSI using γ-H2Ax staining. Contrary to the result of the comet assay, the brightness of sperm DNA from the glass ampoule was significantly higher than those from plastic sheet (Figures 1M–1O; Table S4). These results indicate that the DNA integrity of FD sperm preserved in plastic sheets was comparable to that of conventional glass ampoule spermatozoa.

Table 1. Full term development of embryos derived from freeze-dried sperm preserved in plastic sheet using different materials at −30°C for 1 month

Material	No. of oocytes surviving after ICSI	No. (%) of fertilized embryos	No. (%) of two-cell embryos at 24 h	No. of transferred embryos (no. of recipients)	No. (%) [min-max] of offspring	Mean body weight (g)
Washi	92	87 (95)[a]	60 (69)[a]	60 (4)	5 (8)[a] [0-15]	1.76
Wrap	75	69 (92)	61 (88)[b]	61 (4)	6 (10) [0-56]	1.97
Vinyl sheet	106	87 (82)[b]	78 (90)[b]	78 (6)	16 (21)[b] [9-47]	1.84
Weighing paper	69	59 (86)	48 (81)	48 (4)	10 (21)[b] [0-46]	1.74
Control (Ampoule)	76	63 (83)[b]	51 (81)	51 (3)	12 (24)[b] [20-29]	1.81

ICSI: intracytoplasmic sperm injection; Fertilization rate: number of fertilized embryos/surviving oocytes; Two-cell rate: number of two-cell embryos/fertilized embryos; Offspring rate: number of offspring/transferred embryos. Different letters mean significant difference in each low. Fertilization, two-cell, and the offspring rate were evaluated using chi-squared test. Statistically significant differences between the variables were determined at $p < 0.05$.

Full-term developmental potential is not compromised after preservation

To demonstrate whether the plastic sheet method was applicable to other mouse strains, spermatozoa collected from B6C3F1 male mice were also freeze-dried and injected into ICR oocytes via ICSI. As a result, black-eye offspring were obtained after 1-3 d, 1 week, 1 month, and 3 months of preservation at −30°C (Figure S2A), maintaining the success rate (Figure S2B; Table 2). In addition, randomly selected offspring grew normally until adulthood. Their fertility was normal, and the next generation was also healthy (Figures S2C and S2D). These results indicate that the plastic sheet method can be used to store FD sperm at −30°C regardless of the preservation period and adapted to several strains of mice.

Mouse FD sperm can be mailed via postcard at RT

Finally, we examined whether FD sperm in plastic sheets can be transported at RT. Although the success rate was slightly lower than that obtained from FD sperm preserved at −30°C, as shown in Table 3, the live offspring were obtained from FD sperm preserved for 3 d at RT (Tables 1 and 3). No offspring were obtained after preservation of FD sperm for over 5 d at RT. However, 3 d were sufficient for shipping anywhere within the country. The plastic sheets of FD sperm were put in a single book, such as a card folder, and stored at −30°C freezer (Figures 2A and 2B). The day of mailing, FD sperm sheet was attached on the postcard and attempted to mail it without any special protection (Figures 2C and 2D). After delivery, the FD sperm were stored at −30°C until used in experiments again. Control FD sperm were preserved at the lab bench for the same period. The FD spermatozoa attached to the postcard were sent inside prefecture or from the University of Tokyo to the University of Yamanashi, with a distance of approximately 200 km, and arrived within 2 d. When these FD spermatozoa were used for ICSI, the embryos developed to the 2-cell stage (Figures 2E and 2F) and healthy offspring were obtained after the embryo-transfer into recipient female (Figure 2G), which success rates were comparable to those obtained from the FD control (Figures 2H and 2I; Table 3). Thus, mouse FD sperm could be mailed via postcard at RT without compromising their developmental ability.

DISCUSSION

In this study, we succeeded in preserving the FD sperm on Weighing paper in a thin plastic sheet. When compared to the glass ampoule method, this method is more resistant to losses due to breakage, is cheaper, and less bulkier, which significantly reduces the risk of failure to preserve, preservation costs, and space requirements. If this method is applied for the preservation of mouse strains, tens of thousands of FD sperm can be put in a single "sperm-book" and we can select the samples we need at a glance because there are clear and large sample-names (Figure 2A). This is an extremely efficient method when storing large numbers of genetically modified mouse strains or the conserved genetic resources of wild species.

In the current study, spermatozoa could be stored for more than 3 months at −30°C without decreasing their developmental potential (Table 2), whereas storage at RT was limited to 3 d (Table 3). In our previous

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Table 2. Full-term development of embryos derived from B6C3F1 mouse freeze-dried sperm preserved in plastic sheets at -30°C for up to 3 months

Preservation period	No. of oocytes surviving after ICSI	No. (%) of fertilized embryos	No. (%) of two-cell embryos at 24 h	No. of transferred embryos (no. of recipients)	No. (%) [min-max] of offspring	Mean body weight (g)
1-3 day	151	128 (85)[a]	118 (92)[a]	118 (7)	12 (14)[a] [4-22]	1.63
1 week	147	126 (86)[a]	116 (92)[a]	116 (8)	2 (2)[b] [0-6]	1.93
1 month	203	145 (71)[b]	98 (68)[b]	98 (6)	14 (14)[a] [8-18]	1.58
3 months	182	145 (80)	128 (88)[a]	128 (7)	10 (8)[a] [0-23]	1.63

ICSI: intracytoplasmic sperm injection; Fertilization rate: number of fertilized embryos/surviving oocytes; Two-cell rate: number of two-cell embryos/fertilized embryos; Offspring rate: number of offspring/transferred embryos. Fertilization, two-cell, and the offspring rates were evaluated using chi-squared test. Statistically significant differences between the variables were determined at $p < 0.05$.

study, when we succeeded in FD preservation of mouse spermatozoa for the first time in glass ampoules, the storage at RT was limited to only 1 month (Wakayama and Yanagimachi, 1998). However, recently, we succeeded in storing them at RT for more than a year by improving the vacuum conditions of glass ampoules (Kamada et al., 2018). In this study, after vacuum drying, the FD sperm was exposed to normal pressure and then sealed in plastic sheets. Although sperm are sealed while eliminating as much air as possible, a small amount of air is also contained within the plastic sheets. It is likely that humidity and oxygen contained in this small amount of air had a negative effect on the FD sperm during preservation at RT. On possibility is that Weighing paper was the most suitable for the storage of FD sperm (Figures 1G–1I; Table 1), which may be due to the fact that Weighing paper is the thinnest of all examined materials, the amount of air in the gap of the plastic sheet was small, and sperm oxidation was less likely to occur. Although in the current techniques, the storage period at RT is short, we succeeded in transporting FD sperm between different universities via postcard without any special cold storage equipment or special protection for mailing (Figures 2C, 2D, and 2G; Table 3).

It is now recognized that genetic resources are an asset to humanity's future. Even though many genetic traits are not needed for survival, depending on the environmental context, it is necessary to preserve them as much as possible so that species can survive if unknown diseases are spread or environmental changes, such as global warming, and natural disasters, such as big storm, earthquake, and drought, occur. The plastic sheet preservation method in this study will be the most suitable method for the safe preservation of a large amount of valuable genetic resources because of the resistance to breakage and less space required for storage.

Limitation of the study

In the future, when the preservation period of FD sperm at RT can be extended, this method can facilitate the international use of genetically modified mice due to its extremely low cost. However, the simplicity

Table 3. Full-term development of embryos derived from freeze-dried sperm after preservation at room temperature for 1 to 7 d or after mailing with postcard

Shipment method	Period	No. of oocytes surviving after ICSI	No. (%) of fertilized embryos	No. (%) of two-cell embryos at 24 h	No. of transferred embryos (no. of recipients)	No. (%) [min–max] of offspring	Mean body weight (g)
	1 day	194	165 (85)[a]	139 (84)[a]	139 (7)	10 (7) [0-18]	1.86
None	3 days	167	140 (84)[a]	100 (71)[b]	100 (5)	5 (5) [0-7]	1.99
	1 week	186	114 (61)[b]	66 (58)[c]	66 (3)	0 (0) [0-0]	-
Mail	1-2 day (Inside prefecture)	106	77 (73)	51 (66)[e]	51 (3)	2 (4) [0-14]	1.88
	2 days (Between universities)	100	76 (76)	60 (79)	60 (4)	4 (7) [0-9]	1.89
	2 days (Control)	101	78 (77)	63 (81)[e]	63 (5)	2 (3) [0-17]	1.50

ICSI: intracytoplasmic sperm injection. FD sperm were mailed within Yamanashi Prefecture for 1-2 d, mailed between Chiba Prefecture and Yamanashi Prefecture for 2 d, and as a control, put onto the lab bench at the same time as the mailed postcards. Fertilization rate: number of fertilized embryos/surviving oocytes; Two-cell rate: number of two-cell embryos/fertilized embryos; Offspring rate: number of offspring/transferred embryos. Fertilization, two-cell, and the offspring rate were evaluated using chi-squared test. Statistically significant differences between a vs. b; c vs. d; c vs. e; d vs. e; f vs. g were determined at $p < 0.05$.

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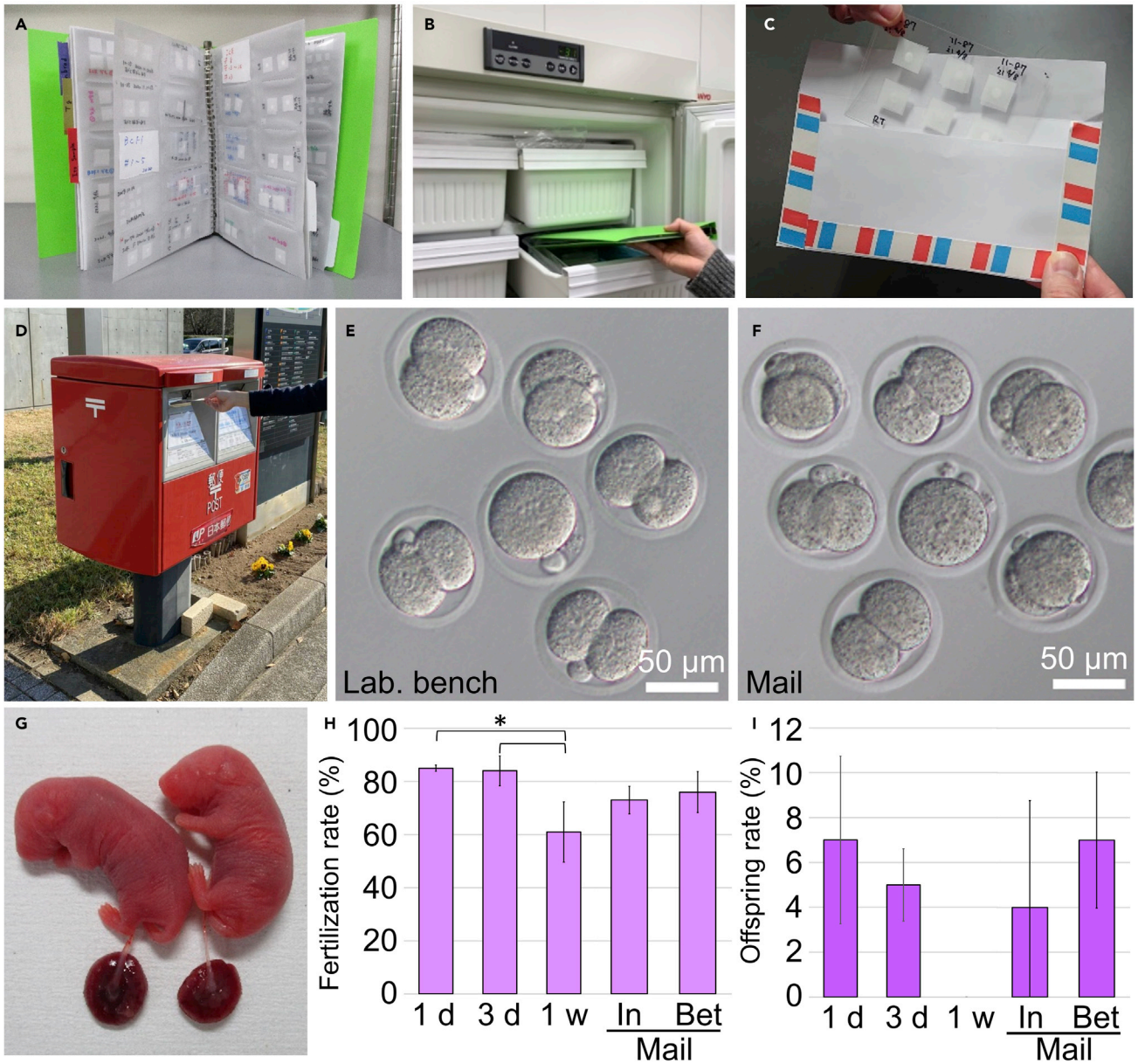

Figure 2. Full-term development of mouse offspring after mailing freeze-dried (FD) sperm by postcard

Full-term development of mouse offspring after mailing FD sperm by postcard FD sperm in the plastic sheet were placed into the card-holder of a book (A), and stored in a freezer at −30°C until further use for the experiments (B). Plastic sheet of FD sperm was attached on the post card or put in the envelope (C), then posted in the mailbox without any protection (D). The 2-cell stage embryos derived from FD sperm preserved at RT for 3 d in laboratory (E) and for 2 d during transportation by postcard (F). Offspring derived from FD spermatozoa after transportation via postcard at room temperature within 2 d (G). The rate of zygote developed to pronuclear stage (H) and full-term developed offspring (I) derived from FD sperm preserved at RT for 1 day, 3 d, and 1 week in the laboratory, and the FD sperm mailed inside the prefecture (In) or between the other prefectures (Bet) by postcard. Significance was determined by the chi-squared test, *: $p < 0.05$.

may increase the illegal transportation of mammalian sperm or genetic resources. Customs and border protections should recognize the potential risk of illegal international transport of germplasm using this method because plastic sheets containing FD sperm will be easy to hide and the current transportation system will not be able to detect the FD sperm and will cause the illicit outflow of genetic resources. Therefore, for the establishment of this sheet preservation technique for FD sperm, it is essential to not only improve the success rate of offspring and extend the preservation period at RT, but also to prepare international laws to prevent their use for purposes other than legitimate purposes, even if this technology was misused.

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STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102815.

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AUTHOR CONTRIBUTIONS

DI, SW, MO and TW conceived and designed the study. DI performed experiments. DI, SW, RE, MO and TW analyzed the data and interpreted the results. DI, SW, MO and TW obtained funding. DI and TW wrote the manuscript. All authors read and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflicts of interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor Cy5-labeled goat anti-rabbit IgG	Abcam	Ab97077
Alexa Fluor 568-labeled goat anti-mouse IgG	Molecular Probes	A-11004
anti-phospho-H2Ax (Ser139) rabbit polyclonal antibody	Millipore-Merck	07-164, UniProt#P16104
anti-histone H3 (dimethyl K9) mouse monoclonal antibody	Abcam	Ab1220
Critical commercial assays		
CometAssay Lysis Solution	funakoshi	4250-050-01
CometAssay LM Agarose	funakoshi	4250-050-02
CometSlide	funakoshi	4250-050-03
DL-Dithiothreitol solution	Sigma-Aldrich	43816-50ML
SYBR® Green	TREVIGEN	4250-050-05
Experimental models: Organisms/strains		
B6C3F1 male mice, 10-12 weeks of age	SLC Inc.	B6C3F1/Slc
ICR female mice, 8-10 weeks of age	SLC Inc.	Slc:ICR
ICR male mice, 10-12 weeks of age	SLC Inc.	Slc:ICR
Other		
Filter paper	Corning	431412
Oblate	Hakujuji Co., Ltd.	JAN code 4987603462489
Plastic sheet	ACCO BRANDS JAPAN K.K.	SLMBCZ
Vinyl sheet	SEISANNIPPONSHA LTD.	D-4
Washi	Boku-Undo Co.,Ltd	28906
Weighing paper	SOGO LABORATORY GLASS WORKS CO.,LTD	4084-03
Wrap	ASAHI KASEI HOME PRODUCTS CORPORATION	JAN code 4901670110388

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents should be directed to and will be fulfilled by the lead contact, Teruhiko Wakayama (twakayama@yamanashi.ac.jp).

Materials availability

All materials in this study are available upon request.

Data and code availability

All data produced in this study are included in this published article and the supplemental information. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Institute of cancer Research (ICR) female mice (8-10 weeks of age and 31-34 g of body weight) (n = 66), ICR male mice (10-12 weeks of age and 38-40 g of body weight) (n = 26), and C57BL/6NCrSlc x C3H/HeSlc (B6C3F1) male mice (8-10 weeks of age and 23-28 g of body weight) (n = 9) were obtained from SLC Inc. (Hamamatsu, Japan). The surrogate pseudo-pregnant ICR females (n = 76), which were used as recipients of the embryos, were mated with vasectomized ICR males (n = 12), whose sterility had been previously demonstrated. On the day of the experiment or after all experiments were completed, the mice were euthanized by CO_2 inhalation or cervical dislocation and were used in the experiments described below. 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 Laboratory Animal Experimentation of the University of Yamanashi, which is followed in the ARRIVE guideline everything.

METHOD DETAILS

Media

HTF medium was used for capacitation (Quinn et al., 1995) and freeze-drying of spermatozoa. CZB medium (Chatot et al., 1990) and HEPES-CZB medium (Kimura and Yanagimachi, 1995) were used for oocyte/embryo incubation in 5% CO2 at 37°C and manipulation, respectively.

Material for holding FD spermatozoa

In this study, we used 6 materials, that is, Traditional Japanese paper, Wrap, Vinyl sheet, Weighing paper, Filter paper, and Oblate. Information were listed on key resources table.

Preparation of FD sperm

We preserved the FD sperm using glass ampoules or plastic sheets (Figure 1A). After the male mouse were sacrificed by cervical dislocation, the epididymis' were collected. Semen was suspended in 750 μl of HTF, and spermatozoa were cultured. All materials (Traditional Japanese paper, Wrap, Vinyl sheet, Weighing paper, Filter paper, and Oblate) were cut into approximately 15 mm squares and placed on plastic sheets, which were also cut into approximately 100 mm squares. Aluminum sheet was floated on top of the liquid nitrogen. After pre-culture of spermatozoa, 50 μl of sperm suspension was dropped onto the Traditional Japanese paper, Wrap, Vinyl sheet, Weighing paper, Filter paper, and Oblate, which were then placed on plastic sheet above the aluminum sheet, and frozen for 10 min. The material with the frozen sperm suspension was placed in a vacuum dryer (FreeZone2.5®, LABCONCO, MO, USA) and dried for 6 h. During the whole drying process, the pressure was 0.100 mBar and the temperature was -50°C inside the vacuum dryer. After the drying process, FD sperm were placed between each material and sealed with glued plastic sheet while removing as much of the air as possible by pressing. The detailed methods are presented in Figure S1. For the control experiment, FD sperms from glass ampoules were prepared as described previously (Wakayama et al., 2017) (Ito et al., 2019). All samples were stored at -30°C or RT (15-25°C) until further use.

Oocyte preparation

Superovulation in female mice was carried out by injecting 5 IU of equine chorionic gonadotropin intraperitoneally, followed by 5 IU of human chorionic gonadotropin after 48 h. Cumulus-oocyte complexes (COCs) were collected from the oviducts of females 14-16 h later and moved to a 35 mm dish containing the HEPES-CZB medium. To disperse the cumulus, COCs were transferred into a 50 μl droplet of HEPES-CZB medium containing 0.1% bovine testicular hyaluronidase for 3 min. Cumulus-free oocytes were washed twice and transferred to a 20 μl droplet of CZB for culture.

Intracytoplasmic sperm injection (ICSI) and embryo transfer

ICSI was performed as described previously (Kimura and Yanagimachi, 1995). Immediately prior to administration of the ICSI, the neck of the ampoule was punctured, 50 μl of sterile distilled water was immediately added to it, and mixed with a pipette. Plastic sheets that preserved FD sperm were cut with scissors, then FD sperm were collected with tweezers into 1.5 ml Eppendorf-tubes, to which 50 μl of sterile distilled water was added immediately and mixed with a pipette. For microinjection of spermatozoa, 1-2 μl of the spermatozoa suspension was moved directly to the drop of polyvinylpyrrolidone on the injection chamber. The spermatozoa suspension was replaced every 30 min during ICSI. Application of several piezo pulses separated the spermatozoa head from the tail, and the head was then injected into the oocyte. The oocytes that

survived ICSI were incubated in CZB medium at 37°C with 5% CO_2. Pronucleus formation was verified 6 h after ICSI. Embryos at the 2-cell stage were transferred to a day-0.5 pseudo-pregnant ICR female mice (body weight: 28-40 g) that had been mated with a vasectomized male the night before transfer (Inoue et al., 2020). Eight to eighteen embryos were transferred into each oviduct. On day 18.5 of gestation, offspring were delivered by cesarean section and allowed to mature. The remaining unused embryos were cultured for up to 4 d to evaluate their potential for development into blastocysts.

Analysis and scoring of comet slides

Spermatozoa DNA damage, potentially caused by single- and double-stranded breaks, was measured using the CometAssay® Kit (Trevigen, MD, USA), according to the manufacturer's instructions. In brief, spermatozoa were collected from ampoules or plastic sheets immediately after opening and rehydrated in water. Specimens and their counterparts were mounted on the same slide, and 100-300 spermatozoa heads on each slide were analyzed by electrophoresis. Three experiments were performed, with the number of measurements being n = 781 for glass ampoules and n = 762 for plastic sheets. To standardize the results across different periods and conditions under which the spermatozoa were stored, the length of each DNA comet tail was divided by the mean length of the one-side results in each experiment. In this comet assay, fresh spermatozoa could not be used as a control because this would require a different preparation technique, which would have prevented a proper comparison between specimens on the same slide.

Gamma-H2Ax assay

Histone H2Ax is an H2A variant. The serine at position 139 of H2Ax is rapidly phosphorylated within seconds of DNA damage. The phosphorylated form of H2Ax, designated as gamma-H2Ax, forms foci at sites of DNA damage, which leads to the recruitment of various repair and cell-cycle checkpoint proteins (Fernandez-Capetillo et al., 2004). Therefore, gamma-H2Ax foci formation was used as a marker of DNA double-strand breaks in male and female pronuclei, and histone H3K9me2 signals were used to distinguish between female and male pronuclei. All specimens were fixed 10 h after ICSI with 4% paraformaldehyde (PFA; Wako Pure Chemical, Osaka, Japan) containing 0.2% Triton X at RT for 20 min and stored in a refrigerator until staining. Primary antibodies used for immunostaining of zygotes included the anti-phospho-H2Ax (Ser139) rabbit polyclonal antibody (1:500; Millipore-Merck, Darmstadt, Germany) and anti-histone H3 (dimethyl K9) mouse monoclonal antibody (1:500; Abcam, Cambridge, UK). The secondary antibodies used were Alexa Fluor 568-labeled goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR, USA) and Alexa Fluor Cy5-labeled goat anti-rabbit IgG (1:500; Abcam). DNA was stained with 4'6-diamidino-2-phenylindole (2 μg/ml; Molecular Probes). The brightness of each male pronucleus was measured using ImageJ software and was subtracted from the brightness of the zygote cytoplasm. Three experiments were performed, with the number of measurements being n = 16 zygotes for glass ampoules and n = 25 zygotes for plastic sheets.

Sperm mailing

After preparation, the FD sperm were stored at -30°C until they were mailed. The FD sperm preserved in the plastic sheets were attached to the postcards or put in the envelope (Figure 2C). After delivery, the sheets were stored again at -30°C until use in experiments (Figure 2B). For the control experiment, some samples were placed on a lab bench at the same time as the other samples were mailed. Mailing was carried out inside Yamanashi Prefecture or between the University of Tokyo (Chiba Prefecture) and the University of Yamanashi (Yamanashi Prefecture).

QUANTIFICATION AND STATISTICAL ANALYSIS

The results of the comet assay and gamma-H2Ax assay were analyzed using the Wilcoxon-Mann-Whitney nonparametric test. Male and female pronuclear formation, *in vitro* development, and the offspring rate were evaluated using chi-squared test. Statistically significant differences between the variables were determined at $p < 0.05$.