

—Technology Report—

Improvement of short straws for sperm cryopreservation: installing an air-permeable filter facilitates handling

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Abstract. Saving space for sperm cryopreservation would aid mouse genetics research. We previously developed the ST (sperm freezing in Short STRaw to reduce Storage space) method for cryopreserving mouse sperm in a smaller storage space than conventional methods. However, our ST method has two drawbacks: difficulties during freeze-thaw procedures and the potential risk of sperm loss during storage. Here, we refine ST, terming the new method improved ST (iST). In iST, the straw has an air-permeable filter and the straw container (2-ml cryotube) is endowed with air vents. As in our ST method, iST frozen-thawed sperm showed good performance upon *in vitro* fertilization. Moreover, up to nine straws can be stored in one cryotube, occupying less storage space than conventional methods. This method provides an easy and space-saving cryopreservation method for mouse sperm, and thus will be valuable for mouse genetics researchers.

Key words: Cryopreservation, *In vitro* fertilization, Mouse, Space-saving, Spermatozoa

(J. Reprod. Dev. 67: 235–239, 2021)

The laboratory mouse (*Mus musculus*) is an essential research animal for studying mammalian biology and human medicine [1]. Many varieties of wild-type and genetically modified mice have been established over the past century [2, 3]. Furthermore, recent advances in genome editing technologies, such as CRISPR and TALEN, have dramatically increased the number of genetically modified mouse lines [4]. These mouse strains will continue to provide valuable insights into the future. For example, previously unexpected results were obtained using genetically modified mouse lines that were generated for other research objectives, such as the discovery of tumor immunotherapy using PD-1-deficient mice [5], generation of iPS cells using Fbx15^{βgeo/βgeo} mice [6], and establishment of a mouse model for intellectual disability using euchromatin histone methyltransferase 1 (*Ehmt1*) heterozygous knockout mice [7]. Thus, maintaining and sharing genetically modified mouse lines in ready-for-use conditions would be valuable for the research community now and into the future. However, maintaining and shipping these mouse lines in a live state requires great labor, financial resources, time, and space [8].

To overcome these limitations, sperm cryopreservation has been a promising strategy for maintaining and shipping mouse genetic resources because of its high reproducibility and technical ease [9]. Although only limited space is required for storing cryopreserved sperm per mouse line, the increasing efficiency of generating geneti-

cally modified mice suggests the need for a more efficient space-saving method of storing cryopreserved sperm in the near future.

Until recently, little attention has been given to the containers used for sperm cryopreservation because the containers currently in use, plastic straws, fulfill most of the demands of researchers [9]. However, plastic straws have several limitations: (1) they are used specifically to cryopreserve sperm and are less common than conventional cryotubes in most laboratories, (2) straws are more fragile than conventional cryotubes, (3) a large amount of space is required to store the plastic straws, and (4) the straws are difficult to store in a box-type liquid N₂ cryogenic storage tank. Further explanations for the limitations (3) and (4) are as follows: a plastic straw has a 1.8 mm outer diameter and is 11.2 cm long, while a cryotube (e.g., Nunc, 366656) has a 12.5 mm outer diameter and is 4.2 cm long. Although the cryotube also requires a large space for storage, the straw requires an even larger space than the cryotube, and it should be stored in a special rack in a liquid N₂ cryogenic storage tank.

We previously developed the ST (sperm freezing in Short STRaw to reduce Storage space) method for cryopreserving mouse sperm in short straws (ST straws hereafter), which dramatically reduced the storage space needed for frozen sperm (less than 16% of the conventional method) [10]. Although the ST straw and method have the benefit of efficiently using storage space, the ST method has some drawbacks, including the need for careful operation during freezing and thawing and the potential loss of sperm-containing straws during storage in a liquid N₂ tank.

Therefore, the present study aims to develop and refine our previous ST method by overcoming the above-mentioned drawbacks. We term the new method the iST method (improved ST). The iST method and the straw used in this method, termed the iST straw (Fig. 1), would be beneficial for the mouse genetic research community.

First, we refined the container to store straw. In our previous ST

Received: February 10, 2021

Accepted: March 28, 2021

Advanced Epub: April 14, 2021

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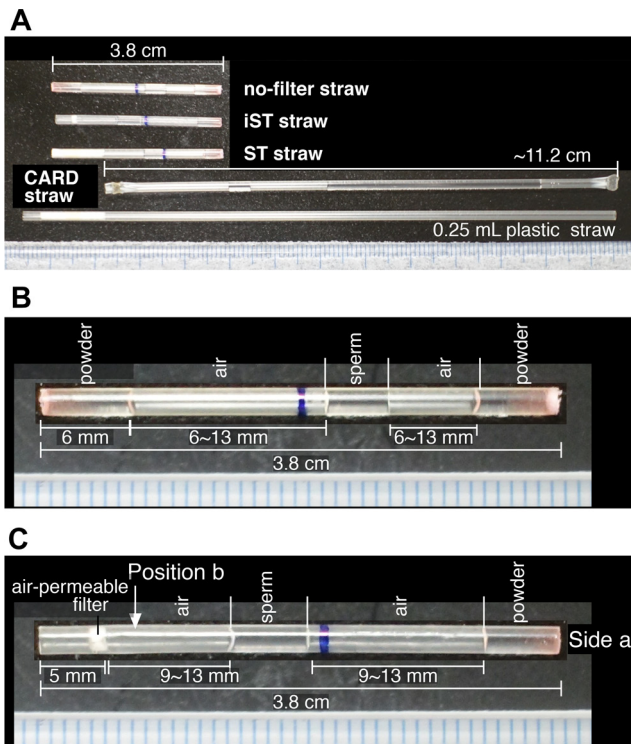


Fig. 1. iST straw and other straws used for sperm freezing. (A) Appearance of straws. A short straw without a filter (no-filter straw), an iST straw, an ST straw, a long straw used for the conventional freezing method (CARD straw), and an unprocessed commercial plastic straw (from top to bottom). (B) Magnified view of no-filter straw. (C) Magnified view of iST straw.

methods, sperm-containing ST straws were stored in a 2-ml cryotube (outer cap type, MS-4603G, Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and the cap of the 2-ml cryotube was loosened and stored in a liquid N_2 tank to lower the risk of cryotube breakage during thawing [10]. However, loosening the cap may increase the risk of the cap being removed, followed by the loss of sperm-containing straws. To overcome this drawback, we opened two holes in the cap of the 2-ml cryotube (Fig. 2A). We found that holes with a diameter smaller than the straw diameter were capable of retaining straws and worked sufficiently as ventilation ducts. Thus, we used a 2-ml cryotube with two holes in the cap as a straw container in this study.

Next, we refined the straw. Our concept was to broaden the working space to lower the potential risk of loss of sperm, which could reduce the care needed during freeze and thaw procedures. Importantly, cryopreserved sperm frozen using the new method should demonstrate comparable performance in *in vitro* fertilization (IVF) to sperm frozen by the conventional CARD method [11] and our previous ST method [10]. In our previous report, sperm frozen using the CARD method and the ST method showed $63.7 \pm 12.8\%$ and $68.5 \pm 15.6\%$ fertilization rates after IVF with C57BL/6J oocytes, respectively [10]. Furthermore, we froze the sperm of several lines of genetically modified mice using the CARD method. Using these frozen sperm, a $55.8 \pm 23.0\%$ fertilization rate was achieved after

IVF with C57BL/6J oocytes (Table 1). These values were used as references for this study.

One side of our previous ST straw contained cotton and powder, which was necessary to seal the straw but markedly reduced the working space. To broaden the working space, we first tried to remove cotton and powder from the ST straw, hereafter the no-filter straw (Figs. 1A and 1B), and examined its performance for sperm cryopreservation. As desired, the risk of sperm loss was reduced compared to our previous ST methods. However, we obtained a significantly lower fertilization rate ($32.3 \pm 6.6\%$) than the values for the CARD and ST methods after IVF with C57BL/6J oocytes (Table 1 and Figs. 3A–3D). The embryos obtained from IVF using sperm frozen by the no-filter method were live born pups practical efficiencies of $30.0\% \pm 18.0\%$, suggesting the embryos have sufficient viability and developmental potency (Table 1). Although the reason for the lower IVF performance is unknown, we argue that the longer handling time required to seal both sides using the straw powder may decrease performance. These results indicate that the no-filter straw and its accompanying method of freezing and thawing were not suitable for sperm cryopreservation.

Finally, we intended to develop a new straw to overcome the limitations of our previous ST straw and the no-filter straw. From the results of the no-filter straw, we speculated that reduction of the handling steps during freezing and thawing would be necessary to maintain sperm quality suitable for IVF. Therefore, we inserted a thin air-permeable filter on one side of the short plastic straw and termed it an iST straw (Fig. 1C). As desired, the working space of the iST straw was broader than that of our previous ST straw, which resulted in easier handling for freezing and thawing than our previous ST methods. Moreover, there were less than half as many handling steps during freezing and thawing compared to using no-filter straw. Notably, no liquid N_2 was observed in the sealed iST straw even after long-term (more than 6 months) storage under liquid N_2 , suggesting that the installed filter prevents liquid N_2 from coming into contact with the sperm solution. Next, the performance of the iST straw was examined. Importantly, a $66.4 \pm 3.6\%$ fertilization rate was achieved after IVF with C57BL/6 oocytes (Table 1 and Fig. 3E), comparable to the values for the CARD method and our previous ST method. Furthermore, the embryos from the iST method gave birth to live born pups at practical efficiencies ($27.2 \pm 20.3\%$), which is comparable with the CARD method, $31.5 \pm 12.1\%$ [10] and $27.9 \pm 18.7\%$ (this study). The efficiencies are also comparable to the efficiency obtained by our previous ST method ($37.5 \pm 6.9\%$) [10]. Although it is now difficult to determine the reason for the improved IVF performance of the iST method compared to the no-filter method, we argue that the shorter handling time or simplicity of the working process when sealing or opening the iST straw may improve the fertilization rate. These results clearly demonstrate that the new iST method is as practical as the conventional CARD method and our previous ST method.

The purpose of the present study was to improve on our ST sperm-freezing method, which dramatically reduced the storage space but contained two weak points. One was difficult to handle during freezing and thawing procedures, and the other was an unexpected cap dropout of the straw container. Here, we optimized two devices (the straw and its container), overcame the two weaknesses, and termed the new

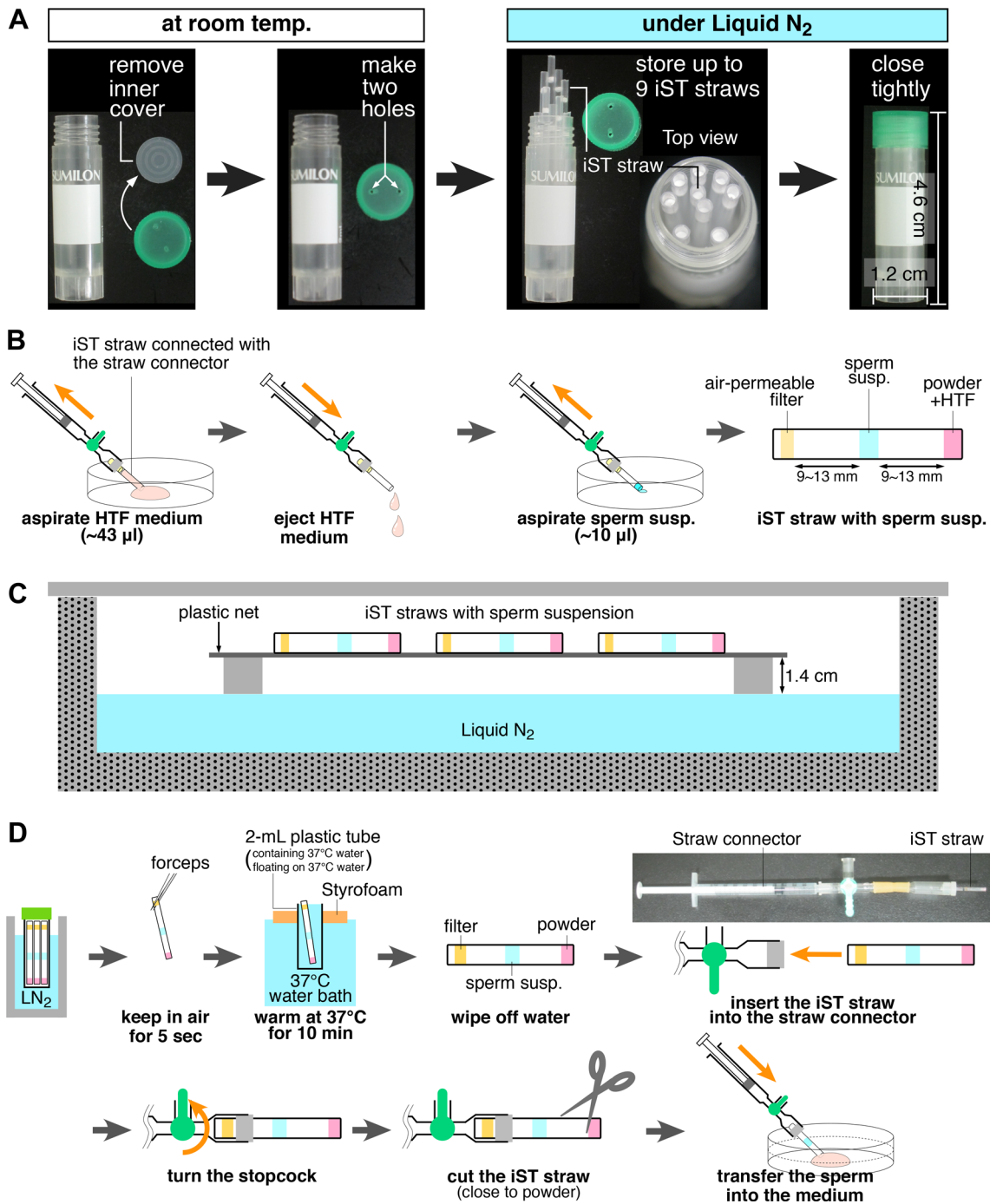


Fig. 2. The freezing, storing and thawing procedures for the iST method. (A) Schematic representation of storing the iST straws in a vial. The iST straws are stored in a vial on which cap two holes are opened, and the vial is stored under liquid N₂. (B) Schematic representation of the procedure for introducing sperm into the iST straw and its sealing. (C) Schematic representation of the freezing procedure for the iST method. (D) Schematic representation of the thawing procedure for the iST method.

method using these devices the iST method. The major advantage of the iST method over the conventional method (i.e., the CARD method) is the reduction in the storage space necessary for frozen sperm. The potential number of iST straws that can be stored in the

same liquid N₂ tank as used in our previous ST method is 6-fold greater than that of the conventional CARD method and is 9-fold greater than that of the cryotube sperm-freezing method developed by Hasegawa *et al.* [12]. Furthermore, the iST and ST straws can be

Table 1. Comparison of *in vitro* fertilization rates and *in vivo* development between two sperm-freezing methods

Method of sperm freezing	Mouse strain Male/Female	<i>In vitro</i> fertilization				<i>In vivo</i> development			References
		No. of exp	No. of females	No. of inseminated oocytes	No. of 2-cell embryos (%) ^a	No. of transferred 2-cell embryos ^b	No. of recipients	No. of live pups (%) ^a	
CARD	B6/B6	21	240	7528	4667 (63.7 ± 12.8) ^c	167	6	53 (31.5 ± 12.1) ^c	Kaneko <i>et al.</i> , 2018
CARD	Tg or KO/B6	16	156	5044	2600 (55.8 ± 23.0) ^c	1037	36	287 (27.9 ± 18.7) ^c	This study
ST	B6/B6	30	94	2619	1799 (68.5 ± 15.6) ^c	109	4	41 (37.5 ± 6.9) ^c	Kaneko <i>et al.</i> , 2018
No-filter	B6/B6	5	16	421	141 (32.3 ± 6.6) ^d	140	5	42 (30.0 ± 18.0) ^c	This study
iST	B6/B6	14	48	1572	1005 (66.4 ± 13.4) ^c	228	9	41 (27.2 ± 20.3) ^c	This study

^a) Results are expressed as the means ± SD. ^b) Twenty to twenty-eight 2-cell embryos per pseudopregnant ICR recipient were transferred. ^c) Values in the same column with a common superscript are not significantly different ($P > 0.05$).

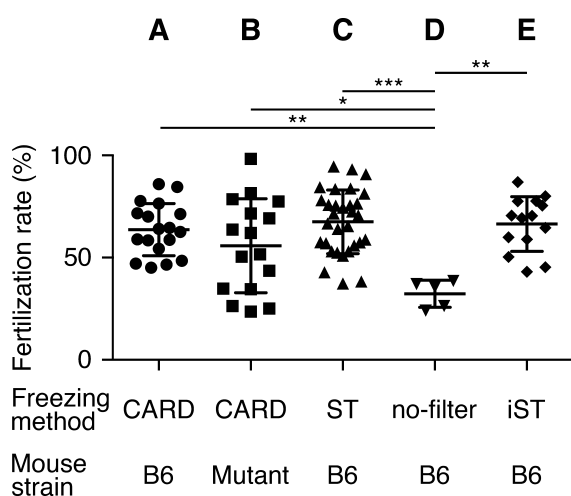


Fig. 3. Rates of *in vitro* fertilization using five types of frozen sperm. Sperm frozen by the following five methods and mouse strains were examined for their viability in IVF: (A) conventional CARD method using B6 mice, (B) conventional CARD method using several gene-modified mouse lines, (C) previous ST method using B6 mice, (D) no-filter method using B6 mice, and (E) iST method using B6 mice. The dots represent individual experiments. The mean and SD are indicated. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

stored in a cryotube, which are more common and less breakable than conventional sperm containers and plastic straws and can be stored in a box-type liquid N_2 cryogenic storage tank. Thus, the present iST method, as well as our previous ST method, can expand the storage capacity of frozen mouse sperm. Therefore, we argue that the present iST method is suitable for freezing mouse sperm with a small storage space and easy handling. Future studies will focus on sperm freezing in other animal species, including human sperm. Furthermore, studies to understand molecular events or genes involved in fertilization or susceptibility to freezing and thawing, as well as to examine the differences between fresh sperm and sperm frozen or thawed by several freezing methods, will improve the quality of frozen-thawed sperm. We hope that these refinements could hopefully establish better methods for sperm cryopreservation [13–15].

In conclusion, we developed the iST method for space-efficient

cryopreservation of mouse sperm and to store its straw in a conventional and less breakable container. Our iST method is easy, reliable, economically feasible, and space-saving. Thus, this method would be valuable not only for individual laboratories, but also for research communities using mouse genetics.

Methods

C57BL/6J mice and B6 albino (B6N-*Tjy*^c-Brd/BrdCrCl) mice were purchased from Japan SLC Inc. (Tokyo, Japan) and Charles River Laboratories Japan, Inc. (Yokohama, Japan), respectively. These mice were used as the sperm and oocyte donors. Female and male donors were 3–4 and 12–15 weeks old, respectively. Mice used as recipients for the transfer of two-cell embryos were of the Slc:ICR strain and were 8–16 weeks old. All animals were maintained under a 12-h/12-h light/dark cycle (lights on: 0600 h to 1800 h) at a constant temperature of 23 ± 1 °C with free access to food and water. All animal experiments were conducted with the approval of the Animal Care and Use Committee of Gunma University (approval No. 16-039).

The media and procedures used for sperm cryopreservation, sperm thawing, IVF, embryo culture, and transfer were essentially the same as those described previously [10, 16].

The sperm were cryopreserved in no-filter straws or iST straws, as described below. After the male mice were sacrificed by cervical dislocation, the two cauda epididymides were collected from one male mouse. The epididymides were incised with fine scissors, and the spermatozoa were allowed to disperse in sperm-freezing medium (100 μ l per two epididymides) (Kyudo Co. Ltd., Kumamoto, Japan). A no-filter straw was prepared as shown in Fig. 1. Briefly, a 0.25-ml plastic straw (IMV, Paris, France) was cut to a length of 3.8 cm, as indicated in Figs. 1A and 1B. Prior to introducing the sperm suspension, ~ 43 μ l of HTF medium (ARK Resource Co. Ltd., Kumamoto, Japan) was aspirated using the CARD straw connector from either side, and then the HTF was ejected from the aspiration side (Fig. 2). Next, the following contents were carefully aspirated: 6–13 mm of air, 10 μ l of sperm suspension, and 6–13 mm of air. After placing these contents in the no-filter straw, both sides were sealed using straw powder (NFA83-6, Fujihira Industry Co., Ltd., Tokyo, Japan) and HTF medium.

The iST straw was produced as shown in Fig. 1. Briefly, a polypropylene tube (outer diameter; 1.8 mm, inner diameter, 1.4 mm)

was cut to a length of 3.8 cm, and then an air-permeable filter (outer diameter, 1.5 mm; pore size, 10 μ m; PTF-100, Wintec Co., Ltd., Niigata, Japan) was inserted on one side by the manufacturer (Joetsu Electronic Ind Co. Ltd., Gunma, Japan) as a custom-made product, as indicated in Figs. 1A and 1C. The introduction of sperm and sealing was carried out as in our previous ST method (Fig. 2B). Prior to introducing the sperm suspension, ~43 μ l of HTF medium (ARK Resource, Kumamoto, Japan) was aspirated using the CARD straw connector from the side without an air-permeable filter (side a) to position b (side a and position b are indicated in Fig. 1C). HTF was then ejected from side a (Fig. 2B). During this procedure, the air-permeable filter should be prevented from becoming wet. Although the rationale for aspirating and ejecting HTF requires further analysis, we predicted that residual HTF or moisture may be beneficial for maintaining sperm performance [10]. Next, the following contents were carefully aspirated: 9–13 mm of air, 10 μ l of sperm suspension, 9–13 mm of air, and 13 μ l of HTF medium. After placing these contents in the ST straw, side a was sealed using straw powder (NEA83-6, Fujihira Industry Co., Ltd.) by introducing the powder into 13 μ l of HTF medium.

The sealed no-filter straws and iST straws were cooled under a liquid N₂ gas layer by laying the straws on a plastic net that was 1.4 cm above the surface of the liquid N₂ (Fig. 2C). After 10 min, the ST straws were plunged directly into liquid N₂. After complete freezing, up to nine no-filter or iST straws were placed in a 2-ml cryotube (outer cap type, MS-4603G, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) using forceps (Fig. 2A), and the vials were stored in a liquid N₂ tank. Two holes were made on the cap by using 24G needle before they contained the straws, as shown in Fig. 2A. The diameter of the hole should be smaller than the diameter of the iST straw (~2 mm). The cap was fastened tightly.

To thaw the spermatozoa, the no-filter straws or iST straws were warmed in a 37°C water bath for 10 min following a 5-sec interval at room temperature (23–25°C) (Fig. 2D). The largest part of the no-filter straw or iST straw containing sperm was immersed in a 37°C water bath. After 10 min of immersion, the no-filter straw or iST straw was removed from the water, and the water was wiped from the straw using fine tissues. The plunger of the CARD straw connector was pulled out of the syringe, and the no-filter straw or iST straw was inserted into the CARD straw connector. Because insertion of the iST straw into the CARD straw connector created pressure inside the iST straw, the stopcock was kept open. The stopcock was then turned to the closed position, and then the ST straw was cut near the powder. The plunger was pushed to transfer the sperm suspension into a drop of FERTIUP medium (Kyudo Co. Ltd.). The media and procedures used for IVF using frozen-thawed sperm of the iST method with glutathione and subsequent embryo culture and transfer were essentially the same as those described previously [16].

Statistical analysis was performed using Prism version 6.0 (GraphPad, Inc., San Diego, CA, USA). Data are shown as the

means \pm SD. Comparison of the differences between the means for each treatment was conducted by analysis of variance after arcsine transformation of the percentage data. Differences between means were considered significant at $P < 0.05$.

Acknowledgments

We acknowledge members of the Bioresource Center, Gunma University Graduate School of Medicine, for providing technical support and participating in helpful discussions. This work was supported by JSPS KAKENHI Grant Numbers 17H05937, 19H04895, and 20H02932 (to RK).

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