Journal of Reproduction and Development, Vol. 64, No 6, 2018

—Technology Report—

Freezing sperm in short straws reduces storage space and allows transport in dry ice

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Abstract. Efficient cryopreservation and transportation of mouse sperm are among the most desirable strategies for current and future research on mouse genetics. However, the current method for sperm cryopreservation uses an 11-cm plastic straw, which is a bulky and fragile container. Developing an alternative to overcome the limitations associated with this method would accelerate biomedical research. Here, we developed the ST (sperm-freezing in ShorT STraw to reduce STorage space) method for cryopreserving mouse sperm in short 3.8-cm plastic straws. Up to nine short straws can be stored in a cryotube, reducing storage space. We further show that sperm frozen by the ST method can be transported in liquid nitrogen or dry ice without any detrimental effects on subsequent fertilization and the birth rate. Our findings suggest that this sperm-freezing method is beneficial not only for individual laboratories but also for large-scale mutagenesis/knockout and phenotyping programs.

Key words: Cryopreservation, *In vitro* fertilization, Mouse, Spermatozoa

(**J. Reprod. Dev. 64:** 541–545, 2018)

An enormous number of genetically modified mouse lines has been generated through genome editing technology and large-scale mutagenesis/knockout and phenotyping programs [1, 2]. Additionally, individual laboratories have generated mice with genetic modifications of specific interest over the past several decades. Furthermore, complex interbreeding of several genetically modified mouse lines has been widely conducted in individual laboratories, such as the production of conditional knockout mice harboring a floxed allele and Cre expressing unit. Thus, the financial resources, time, and space needed to maintain these mouse lines are continuing to increase.

To overcome these limitations, the cryopreservation of sperm has become an increasingly promising strategy for maintaining mouse genetic resources because of its high reproducibility and technical ease [3]. Increasing amounts of cryopreserved sperm are stored in international bioresource banks, such as the Jackson Laboratory, Mutant Mouse Resource & Research Center, European Mouse Mutant Archive, and RIKEN BioResource Center, as well as in individual laboratories. Furthermore, to prevent accidental loss of these genetic resources, it would be advantageous to preserve sperm in at least two facilities. Therefore, an unlimited storage capacity for cryopreserved mouse sperm would be required.

Until recently, little attention has been paid to the containers used for cryopreserving mouse sperm because the plastic straws currently used demonstrate good performance [3]. However, these straws have several limitations: (1) These straws are used specifically to cryopreserve sperm and are less common than conventional cryotubes in most laboratories. (2) The straws are more fragile than conventional cryotubes. (3) A large amount of space is required to store the plastic straws. Although Hasegawa *et al.* recently solved the first and second problems by developing a protocol to cryopreserve mouse sperm in cryotubes [4], the third problem remains to be solved.

Here, we developed the ST (sperm-freezing in ShorT STraw to reduce STorage space) method for cryopreserving mouse sperm in short straws to reduce storage space. We also demonstrated that sperm frozen by the ST method can be transported in liquid nitrogen (LN_2) or dry ice.

We first optimized a method for cryopreserving mouse sperm using the ST straw (Fig. 1) by applying several modifications to the CPA-LGlu protocol, which is referred to as the CARD method hereafter [5]. The aim of this experiment was to develop a sperm-freezing method using ST straws with comparable performance of sperm in *in vitro* fertilization (IVF) to that of sperm preserved by the conventional CARD method. Using sperm frozen by the CARD method, a 63.7 ± 12.8% fertilization rate was achieved after IVF with C57BL/6J oocytes (Table 1 and Fig. 2A). After several optimizations, we achieved a 68.5 ± 15.6% fertilization rate using sperm frozen in ST straws (Table 1 and Fig. 2B). Furthermore, the embryos from both methods gave birth to liveborn pups with comparable efficiencies (37.5 ± 6.9% with the ST method and 31.5 ± 12.1% with the CARD method) (Table 1). These results demonstrated that our ST method is as practical as the conventional CARD method.

Next, we determined how each procedure optimized for the ST method affected the fertilization rate in IVF. Three types of frozen sperm that had been all frozen by the ST method but with one procedure varied from our optimized method were thawed and used for IVF. As shown in Fig. 2C, the fertilization rate was significantly affected by the freezing apparatus (44.8 ± 19.0%). As shown in Fig. 2E, we omitted the procedures of aspirating and ejecting human

Received: August 21, 2018
Accepted: September 11, 2018
Published online in J-STAGE: September 28, 2018

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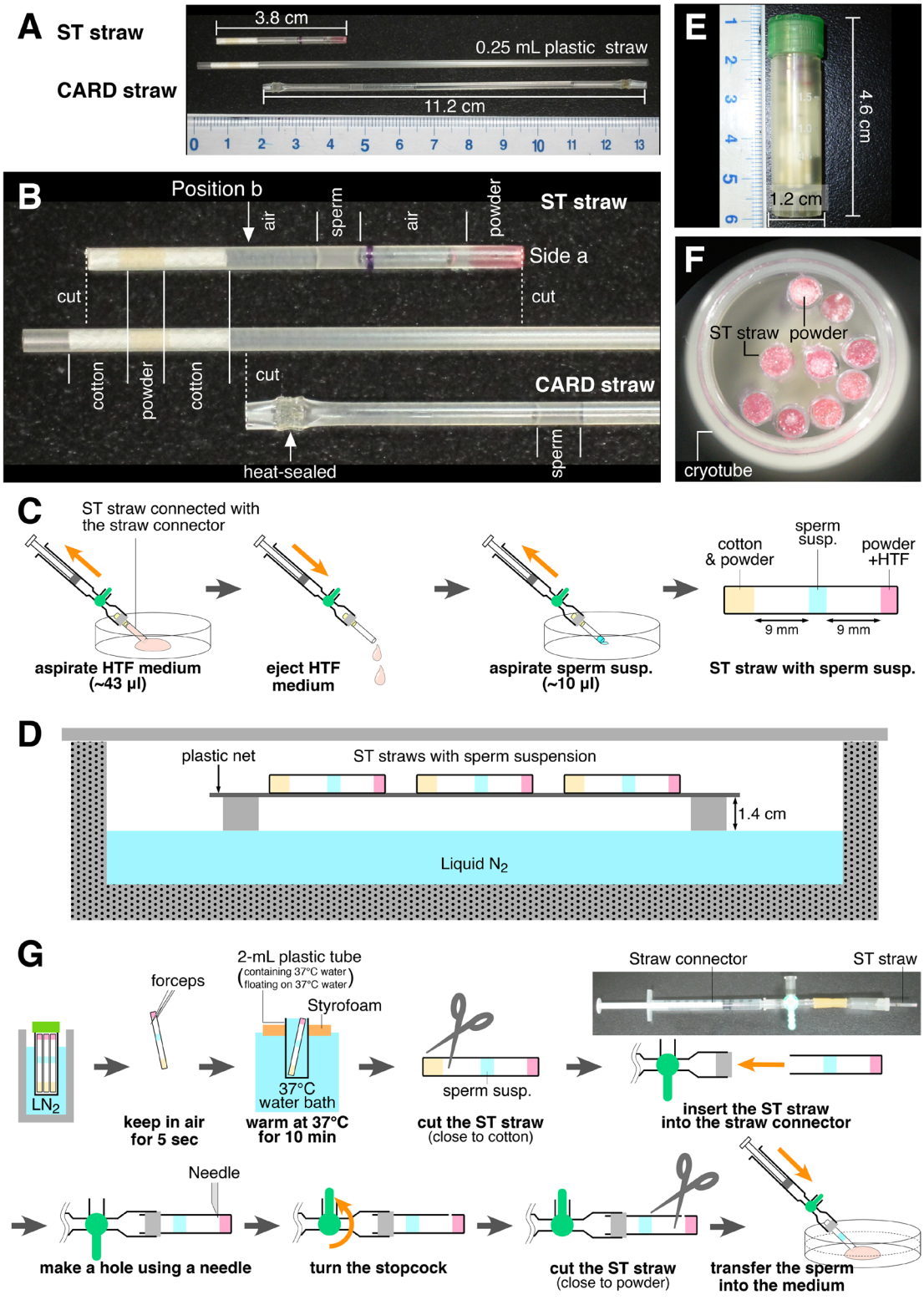

Fig. 1. ST straws and the freezing and thawing procedures for the ST method. (A) Appearance of straws. An ST straw (top), an unprocessed commercial plastic straw (middle) and long straw used for the conventional freezing method (CARD method) (bottom). (B) Magnified view of straws. The ST straw and CARD straw were prepared by cutting the commercial straw at the indicated positions. (C) Schematic representation of the procedure for introducing sperm into the ST straw and its sealing. (D) Schematic representation of the freezing procedure for the ST method. (E) Side view of the outer cap-type cryotube containing 9 ST straws. (F) Top view of the outer cap-type cryotube containing 9 ST straws. To show the inside of the cryotube, the cap was removed for this picture. (G) Schematic representation of the thawing procedure for the ST straw.

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

Method of sperm freezing	*In vitro* fertilization				*In vivo* development		
	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	21	240	7528	4667 (63.7 ± 12.8) [c]	167	6	53 (31.5 ± 12.1) [c]
ST	30	94	2619	1799 (68.5 ± 15.6) [c]	109	4	41 (37.5 ± 6.9) [c]

[a)] Results are expressed as the means ± SD. [b)] Twenty to 28 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).

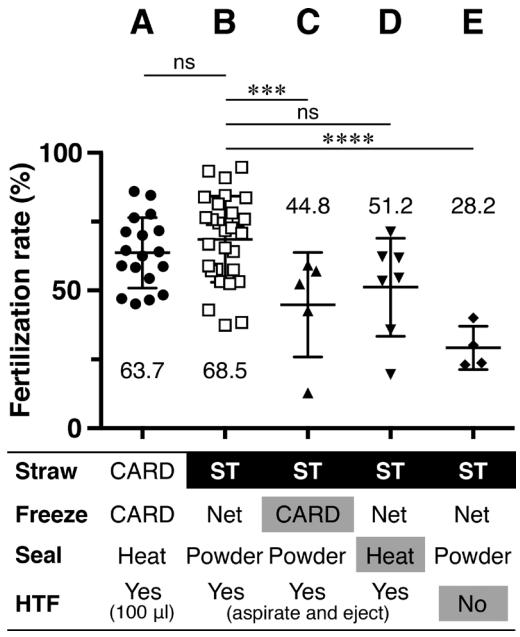

Fig. 2. Rates of *in vitro* fertilization using five types of frozen sperm. Sperm frozen by the following five methods were examined for their IVF ability: (A) conventional CARD method, (B) ST method, (C) altered ST method (sperm frozen using the same equipment as for the CARD method), (D) altered ST method (straws sealed using a heat sealer), and (E) altered ST method (sperm introduced without aspiration and ejection of HTF). The numbers represent mean fertilization rate (%). The dots represent individual experiments. The mean and SD are indicated. *** P < 0.001; **** P < 0.0001.

tubal fluid (HTF) medium prior to loading of the sperm (the first and second panel from the left in Fig. 1C), which greatly affected the fertilization rate (28.2 ± 7.9%). Although not significant, sealing the straws using a heat sealer slightly lowered the fertilization rate (51.2 ± 17.8%) (Fig. 2D). Furthermore, sealing with powder requires less labor and is more reliable than using a heat sealer. These results indicate that combining the three optimized procedures results in satisfactory performance of the ST method.

Finally, to confirm that sperm frozen by the ST method could be transported between laboratories, the frozen sperm were transported and then their performance in IVF and successful generation of live pups were examined. Furthermore, we tested whether shipment of the frozen sperm in dry ice had adverse effects on the samples. Sperm from C57BL/6J male mice were frozen by our ST method. Four to six straws for each method were placed in a cryotube, shipped in LN_2 dry shippers or in Styrofoam boxes filled with dry ice from our facility to another facility, and then returned to our facility (total distance of transportation was ~1,000 km, and the shipment required 2 days). The results of this transportation study are summarized in Table 2. Each frozen-thawed sperm successfully generated 2-cell embryos by IVF. In both cases, transport of frozen sperm in ST straws in LN_2 or dry ice did not significantly affect the fertilization rates (63.4 ± 17.6% in LN_2 and 71.7 ± 8.1% in dry ice). These embryos were successfully implanted into recipient females, which gave birth to liveborn pups (24.8 ± 21.7% in LN_2 and 22.4 ± 11.6% in dry ice) with a comparable efficiency as non-transported sperm (see Table 1). The body weight of the pups at 2 weeks of age was not significantly different between pups obtained by IVF using frozen sperm transported in LN_2 (male: 9.5 ± 1.4 g, female: 9.3 ± 1.2 g) and those obtained by IVF using frozen sperm transported in dry ice (male: 9.6 ± 0.8 g, female: 9.6 ± 1.6 g) (mean ± SD). Moreover, another generation was successfully obtained by mating males and females generated by IVF using sperm frozen by the ST method (data not shown). Taken together, sperm frozen by the ST method

Table 2. Comparison of *in vitro* fertilization rates and *in vivo* development between two transport conditions

Conditions of transport	*In vitro* fertilization				*In vivo* development		
	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)]
LN_2 dry-shippers	3	23	629	398 (63.4 ± 17.6) [c]	201	9	49 (24.8 ± 21.7) [c]
Dry ice	3	23	716	498 (71.7 ± 8.1) [c]	324	13	73 (22.4 ± 11.6) [c]

[a)] Results are expressed as the means ± SD. [b)] Twenty to 28 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).

can be transported without detrimental effects.

The purpose of the present study was to improve the sperm-freezing method to reduce the necessary storage space and ease of transportation. We optimized several procedures and developed the ST method for cryopreserving mouse sperm in short straws. This study involved a series of two experiments. In the first experiment, we developed a method for cryopreserving mouse sperm in short straws (named as ST straws) and compared the success of this sperm in IVF to that of sperm preserved by the conventional freezing method. We also examined the effects of different procedures used in the ST method on its success. The second experiment was carried out to confirm that sperm frozen by the ST method could be transported between laboratories in LN_2/dry ice without lowering fertilization efficiency.

The major advantage of our ST method is the reduction in the storage space necessary for frozen sperm. The potential number of straws stored in a liquid nitrogen tank can be estimated as follows. The MVE XC series (Chart MVE BioMedical, Ball Ground, GA, USA) liquid nitrogen tanks are used as examples. When using an XC 47/11-6 tank (cane type, LN_2 capacity of 47.4 l), the maximum number of CARD straws is 2,376 (264 canes, 9 straws/cane), while that of ST straws is 14,256 (264 canes, 6 vials/cane, 9 straws/vial). When using the XC 47/11-SQ tank (box type, LN_2 capacity of 47.4 L), no CARD straws can be stored because of insufficient space, but the tank can store 6,750 ST straws (30 boxes, 25 vials/box, 9 straws/vial). Furthermore, a larger tank (MVE CryoSystem 6000, LN_2 capacity of 175 L) can store 54,000 ST straws (60 boxes, 100 vials/box, 9 straws/vial). Additionally, Hasegawa *et al.* recently reported a method for freezing sperm in cryotubes [4]. In this method, one cryotube contains 10 µl of sperm suspension. In contrast, 9 ST straws can be stored in one cryotube, resulting in a 9-fold greater storage capacity than the method developed by Hasegawa *et al*. Thus, the ST method can expand the storage capacity for frozen mouse sperm.

The ST method may also be applied for promoting the distribution of mouse sperm. Although a recent study reported that frozen mouse sperm can be transported in dry ice without losing fertility [6], the most common transport method currently used by the scientific community is shipment in LN_2 using dry shippers. Shipping in dry ice has several advantages compared to shipping in LN_2: it is safer, does not require a special dry-shipper container, and can be conducted in a Styrofoam box, which does not need to be returned to the sender. Moreover, dry ice is less costly than LN_2. Our results strongly support that dry ice is a reliable substrate for distributing mouse sperm [6] and demonstrate that sperm frozen by the ST method are suitable for shipping in dry ice.

During sperm transportation, the sperm containers may be broken. Although the sperm freezing protocol in cryotubes lowered this risk [4], the most common freezing protocol currently uses long straws as sperm containers. To safely transport the long straws, a special container (such as a triangular cassette, REF. 1981/01201, Minitube USA, Delavan, WI, USA) may be required. In contrast, the ST straw can be transported in cryotubes, which are harder than long straws and more common than the triangular cassette.

We examined the sperm of only one wild-type mouse strain, C57BL/6J, in this study. Although C57BL/6J is a standard inbred strain of genetically modified mouse, other strains are also used; C57BL/6N is used by the International Mouse Phenotyping Consortium, FVB/N is used by the Gene Expression Nervous System Atlas project, and various strains are used by each laboratories for specific purposes [1, 7]. Previous studies reported that the fertilizing ability of frozen-thawed sperm varies between mouse strains (low for C57BL/6 and high for DBA/2) [8–10]. Thus, future studies should examine the applicability of the ST method to other wild-type strains and mutant mouse strains. Furthermore, sperm cryopreservation has become widely used since the 1970s for humans and animals, causing the amount of frozen sperm to continue to increase [11–13]; therefore, improving sperm containers is very important.

In conclusion, we developed the ST method for cryopreserving mouse sperm in small spaces and transporting these samples in LN_2 or dry ice. Our ST method is easy, reliable, and space-saving. Thus, this method may be beneficial not only for individual laboratories but also for large-scale mutagenesis/knockout and phenotyping programs.

Methods

C57BL/6J mice were purchased from Japan SLC Inc. (Tokyo, Japan) and used as 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 dark/light 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 carried out with the approval of the Animal Care and Use Committee of Gunma University.

The media and procedures used for sperm cryopreservation, sperm thawing, IVF, embryo culture, and transfer were essentially the same as those described previously [14].

The sperm was cryopreserved in ST 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 µl per two epididymides) (Kyudo, Kumamoto, Japan). The ST 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 Fig. 1A & 1B. Prior to introducing the sperm suspension, ~43 µl of HTF medium (ARK Resource, Kumamoto, Japan) was aspirated using the CARD straw connector from the side without cotton (side a) to position b (side a and position b are indicated in Fig. 1B), and then the HTF was ejected from side a (Fig. 1C). During this procedure, the cotton was prevented from becoming wet. Although the rational of this procedure requires further analysis, we predicted that the residual HTF or moisture may be beneficial for maintaining sperm performance (see Fig. 2E). Next, the following contents were carefully aspirated: 9 mm of air, 10 µl of sperm suspension, 9 mm of air, and 13 µl of HTF medium. After placing these contents in the ST straw, side a was sealed using straw powder (NFA83-6, Fujihira Industry, Tokyo, Japan) by introducing the powder into the 13 µl of HTF medium. The sealed ST straws were cooled under an LN_2 gas layer by laying the straws on a plastic net which was 1.4 cm above the surface of the LN_2 (Fig. 1D). After 10 min, the ST straws were plunged directly into LN_2. After complete freezing, up to nine ST straws were placed in a 2-ml cryotube (outer cap type, MS-4603G,

Sumitomo Bakelite, Tokyo, Japan) using forceps (Fig. 1E and 1F), and the vials were stored in an LN_2 tank. Note that do not fasten the caps too tightly while storing in the LN_2 tank.

To thaw the spermatozoa, the ST straws were warmed in a 37°C water bath for 10 min following a 5-sec interval at room temperature (23–25°C) (Fig. 1G). The largest part of the ST straw containing the sperm was immersed in the water bath. After 10 min of immersion, the ST straw was removed from water, and then the water was wiped from the ST straw using fine tissues. The plunger of the CARD straw connector was pulled out of the syringe, and the ST straw was cut close to the cotton. The cut ST straw was inserted into the CARD straw connector. Because insertion of the ST straw into the CARD straw connector created pressure inside the ST straw, the position of the stopcock were kept in the open position. To further reduce the pressure inside the ST straw, a small hole was made with a 27-gauge needle as close as possible to the powder. The stopcock was 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 the drop of FERTIUP medium (Kyudo, Kumamoto, Japan). The media and procedures used for IVF using the frozen-thawed sperm of the ST method with glutathione, and subsequent embryo culture, and transfer were essentially the same as those described previously [5, 14].

To transport the frozen sperm, the ST straws containing cryopreserved sperm were placed into a 2-ml cryotube (outer cap type, MS-4603G, Sumitomo Bakelite). The cryotubes containing ST straws were then transported under one of two conditions (in an LN_2 gas layer using a dry shipper or in dry ice using a Styrofoam box). Four to six straws from each method were sent from our facility (Gunma, Japan) to another facility (Osaka, Japan; ~500 km away from our facility by land transportation) using a common courier company. Upon arrival at the other facility, the straws were sent back to our facility using a similar method (~1,000 km total distance over 2 days). The straws were stored in an LN_2 tank for several days and then thawed and used for IVF of C57BL/6J oocytes.

Statistical analysis was performed using Prism version 6.0 (GraphPad, San Diego, CA, USA). Data are shown as the means ± SD. Comparison of the differences between the means for each treatment was carried out by analysis of variance after arcsine transformation of the percentage data. Differences between the means were considered as significant when $P < 0.05$.

Acknowledgments

We acknowledge Drs Etsuko Tarusawa, Ayako Morita and members of the Bioresource Center, Gunma University for providing technical support and participating in helpful discussions. This work was supported by JSPS KAKENHI Grant Numbers 15K06696, 15KK0331, and 17H05937 (to RK).

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