Optimization of a Protocol for Cryopreservation of Mouse Spermatozoa Using Cryotubes

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Abstract. The rapid increase in the number of genetically modified mouse strains has produced a high demand for their frozen spermatozoa from laboratories and mouse banking facilities. Historically, plastic straws have been used preferentially as containers for frozen mammalian spermatozoa because spermatozoa frozen in plastic straws have a high survival rate after thawing. However, plastic straws are more fragile and are used less often than the cryotubes used for conventional cell freezing. In this study, we sought to develop a new protocol for sperm freezing using cryotubes as the container to increase the accessibility of mouse sperm cryopreservation. Epididymal spermatozoa were collected from mature ICR or C57BL/6J (B6) males and were suspended in 18% raffinose and 3% skim milk solution. We then optimized the following conditions using the sperm survival rate as an index: 1) distance of cryotubes from the surface of the liquid nitrogen at freezing, 2) volume of the sperm suspension in the cryotube and 3) temperature of warming sperm during thawing. The best result was obtained when cryotubes containing 10 µl of sperm suspension were immersed 1 cm below the surface of the liquid nitrogen and then thawed at 50°C. The fertilization rates using spermatozoa frozen and thawed using this method were 63.1% in ICR mice and 28.2% in B6 mice. The latter rate was increased to 62.3% by adding reduced glutathione to the fertilization medium. After embryo transfer, 68% and 62% of the fertilized oocytes developed into normal offspring in the ICR and B6 strains, respectively. These results show that cryotubes can be used for cryopreservation of mouse spermatozoa under optimized conditions. This protocol is easy and reproducible, and it may be used in laboratories that do not specialize in sperm cryopreservation.

Key words: C57BL/6, Glutathione, Mouse, Spermatozoa

Large-scale mutagenesis/knockout and phenotyping programs using mouse strains have been launched to provide the research community with a long-lasting resource for the study of mammalian gene function [1, 2]. In addition to these large organized programs, individual laboratories generate mice with gene modifications of specific interest using conventional transgenic and knockout methods [3, 4]. These uses have increased the demand for the efficient and safe preservation of invaluable mouse strains. Since the first success by Whittingham in 1972 [5], embryo cryopreservation has been the main strategy for preserving mouse genetic resources because of its high reproducibility and technical ease.

From the practical viewpoint, however, preservation of mouse strains as embryo stocks has several technical disadvantages because of the steps required before the production of embryos, including superovulation of females, in vitro fertilization (IVF), and in vitro embryo culture. All of these steps should be well controlled for the best results. In addition, the number of oocytes collected from a single female is limited to 5.4–39.5, depending on the strains used [6]. To circumvent these drawbacks associated with embryo cryopreservation, sperm cryopreservation is now used extensively as a more promising strategy for mouse strain preservation because it is quick and can produce a great number of genetic materials from a single male animal [7, 8].

In general, use of one sexually mature male is enough to produce a mouse strain stock when the strain carries a single or limited number of mutant genes under a defined genetic background [9]. The sperm nucleus is more robust than the embryo nucleus and may survive accidental breakdown of temperature control [10]. Normal pups have been produced by injecting oocytes with spermatozoa retrieved from a whole-mouse body frozen conventionally for 15 years [11]. This result shows that the mouse sperm nucleus is physically much stronger than expected.

To produce frozen sperm stocks for routine use in the derivation of mouse lines, thawed spermatozoa should be intact to maintain the high motility necessary for successful IVF. This has been one of the major technical challenges associated with sperm cryopreservation since the first success about 20 years ago [12, 13]. The sperm
membrane, especially around the acrosome, is very sensitive to freezing–thawing procedures, and injury to the membrane can decrease the fertilization rate following IVF [14]. Chilling, hypertonic stress and intracellular ice are considered the major causes of sperm injury and may occur through different pathways [15]. Mechanical damage to the sperm membrane occurs most often in spermatozoa from the C57BL/6 (B6) strain, one of the standard inbred stains of genetically engineered mice. This has prompted several technical improvements for cryopreservation of B6 spermatozoa by modifying the composition of the cryopreservation solution or the sperm preincubation medium [16–19].

Little attention has been given to the containers used for freezing spermatozoa because the plastic straws currently used are generally satisfactory. However, these straws are used specifically for sperm freezing and are less familiar than conventional cryotubes in most laboratories. The demand for freezing spermatozoa in cryotubes has increased with the use of genetically modified mice in various laboratories because cryotubes are harder than plastic straws and are more suitable for transporting samples. Furthermore, cryotubes are easy to handle and commercially available in any laboratory worldwide. To date, spermatozoa from CBA [20], B6D2F1 [21] and B6129SF1 [22] have been successfully cryopreserved in cryotubes for later IVF experiments, but the technique is still impractical for spermatozoa from several strains including B6 [23, 24].

The present study was undertaken to develop a method for sperm cryopreservation using cryotubes. Several factors that may affect the survivability of spermatozoa were optimized based on analysis of variance (ANOVA) of two major sperm parameters: the rate of motile spermatozoa and the rate of progressively motile spermatozoa. Epididymal spermatozoa collected from ICR and B6 males were used in the experiments.

The cooling rate during the freezing of samples may vary with the distance from the surface of liquid nitrogen (LN2). We first examined the effect of the position of the cryotube during freezing on the survival of spermatozoa. In this experiment, the volume of sperm suspension in the cryotube was set at 10 µl, and the frozen cryotubes were thawed at 37°C following our conventional sperm cryopreservation protocol using plastic straws [25, 26]. The position of the cryotubes, but not the mouse strain, had a significant effect on sperm survival, and there was no interaction between the factors (position of cryotubes and mouse strain) (Table 1). The best sperm survivability was obtained when the bottom of the cryotube was immersed 1 cm under the surface of LN2, although the efficiency was still lower than that in the fresh and straw-freezing groups (Fig. 1a).

We next examined whether the volume of the sperm suspension (1, 10 or 50 µl) inside the cryotube affects sperm survival. Both factors (the mouse strain and the volume of the sperm suspension) significantly affected sperm survival, but there was no interaction between the factors (Table 1). In the cryotube groups, which were frozen at 1 cm under the surface of LN2, the highest survival parameters were obtained in the 10 µl group, and these parameters did not differ significantly from those in the plastic straw groups (Fig. 1b). ICR sperm showed better survival parameters than B6 sperm (Fig. 1b).

Finally, we sought to optimize the conditions for sperm thawing. Both factors (the mouse strain and the method of warming) had significant effects on sperm survival, and there was a significant interaction between these factors in the rate of motile spermatozoa but not the rate of progressively motile spermatozoa (Table 1). This indicates that the rate of motile spermatozoa was determined by the combinations of the mouse strain and the method of warming, while the rate of progressively motile spermatozoa was affected by these factors separately. Compared with the freezing conditions tested above, the thawing protocols had fewer significant effects on the survivability of spermatozoa. Most significant differences were found between the fresh sperm group and the freezing–thawing groups. Among the cryotube-freezing groups, those thawed at 50°C consistently produced the best sperm motility, although there was no statistical significance (Fig. 1c). The rate of motile spermatozoa was higher in sperm from ICR mice than in sperm from B6 mice. Therefore, for subsequent experiments, we used the 50°C condition for warming cryotubes starting 30 s after their retrieval from LN2. During this time, the LN2 inside the cryotube was allowed to evaporate, and the sperm suspension thawed completely in about 1 min. We also examined the third motility parameter for spermatozoa, track speed. Although both factors (the mouse strain and the method of warming) had significant effects on the track speed, there was no interaction between the factors (Table 1). There was no significant difference between the cryopreservation groups, including the plastic straw group (Fig. 2). ICR spermatozoa showed significantly better results than did B6 spermatozoa (Fig. 2).

As described above, the motility of spermatozoa could be restored after thawing when the sperm were cryopreserved precisely in cryotubes. In the next series of experiments, we examined the feasibility of this optimized protocol when used for IVF using frozen–thawed spermatozoa. We used the following sperm cryopreservation protocol. Cryotubes containing 10 µl of sperm suspension were immersed 1 cm below the surface of the LN2, and the cryotubes were thawed at 30°C following a 30-sec interval at room temperature. The oocytes were collected from females of the same strain as the spermatozoa (ICR and B6). Spermatozoa freshly collected or frozen in plastic straws were used as the controls. The spermatozoa were preincubated in a medium containing methyl-β-cyclodextrin, as reported previously [17, 18]. As shown in Fig. 3a, the average fertilization rate did not differ between spermatozoa frozen in cryotubes and spermatozoa frozen in plastic straws. However, the fertilization rates were significantly lower in the B6 group than in the ICR group, especially when frozen–thawed spermatozoa were used (28.2% with straw-frozen, 24.0% with cryotube-frozen and 74.1% with fresh spermatozoa). To increase the fertilization rates when using frozen B6 spermatozoa, we added reduced glutathione (GSH) to the IVF medium because this chemical increases the IVF efficiency by decreasing reactive oxygen species from damaged spermatozoa that may inhibit fertilization [27]. The fertilization rates increased to 75.0% and 69.3% with spermatozoa frozen in straws and cryotubes, respectively; these values were similar to those obtained with fresh spermatozoa (Fig. 3b). ICR spermatozoa were more tolerant to the freezing and thawing procedures than B6 spermatozoa, and therefore, use of GSH did not result in further improvement of the fertilization rates (70.4 ± 10.1% vs. 63.1 ± 7.0%;
**Fig. 1.** The effects of the freezing and thawing conditions on the viability of spermatozoa. 

**a:** The effect of the position of the cryotube during cooling. The highest motility of spermatozoa among the cryotube groups was obtained when the cryotube was cooled with its bottom 1 cm under the surface of the liquid nitrogen (–1 cm) (a, a’ P < 0.05). The sperm suspension volume was 10 µl, and the thawing temperature was 37 °C throughout the experiments. Mean ± SEM, n=4–5 per group. 

**b:** The effect of the volume of sperm suspension. The highest motility of spermatozoa after thawing was observed when 10 µl of sperm suspension was frozen in the cryotubes, being comparable to that of the straw-frozen group (a, a’, b, b’ P > 0.05). The freezing cryotubes were positioned 1 cm below the surface of the liquid nitrogen, and the thawing temperature was 37 °C throughout the experiments. Mean ± SEM, n=4–5 per group. 

**c:** The effect of the thawing temperature. Unlike the cooling conditions shown above, the thawing conditions did not have a significant effect on sperm survival between the cryotube-frozen groups. a, a’ P < 0.05. There were always significant differences between the fresh sperm group and other frozen sperm groups (not indicated). For the corresponding ANOVA analysis data, see Table 1. Mean ± SEM, n=5–7 per group.

**Table 1.** Probabilities (P values) of main effects on sperm parameters and their interaction

| Corresponding experiment (figure) | Effect and interaction | Rate of motile spermatozoa | Rate of moving spermatozoa |
|-----------------------------------|------------------------|-----------------------------|----------------------------|
| Fig. 1a                           | Main effect            | Strain                      | 0.742                      | 0.589                      |
|                                    |                        | Position of cryotube at freezing | 0.000                      | 0.000                      |
|                                    |                        | Interaction between two factors | 0.428                      | 0.661                      |
| Fig. 1b                           | Main effect            | Strain                      | 0.020                      | 0.035                      |
|                                    |                        | Volume of the sperm suspension | 0.000                      | 0.000                      |
|                                    |                        | Interaction between two factors | 0.776                      | 0.608                      |
| Fig. 1c                           | Main effect            | Strain                      | 0.000                      | 0.000                      |
|                                    |                        | Warming temperature         | 0.000                      | 0.000                      |
|                                    |                        | Interaction between two factors | 0.036                      | 0.143                      |
| Fig. 2                            | Main effect            | Strain                      | 0.000*                     |                             |
|                                    |                        | Warming temperature         | 0.000*                     |                             |
|                                    |                        | Interaction between two factors | 0.894*                     |                             |

Results were obtained by two-way ANOVA analysis. A probability of P<0.05 was considered significant (underlined). *Track speed.
P<0.05). We performed an embryo transfer experiment to confirm the normality of the embryos produced; the implantation and birth rates were 85% (45/53) and 68% (36/53) for the ICR strain and 89% (47/53) and 62% (33/53) for the B6 strains.

It is generally accepted that spermatozoa survive better after freezing–thawing in plastic straws than in cryotubes [23, 24, 28], possibly because the wall of a straw is thinner than that of a conventional cryotube. However, with increasing demand for protocols using cryotubes, which are more accessible and physically stronger than plastic straws, several technical protocols for

Fig. 2. The effect of the thawing temperature on the track speed of spermatozoa. Track speed did not differ significantly between the cryotube-frozen groups. *a,a*′P<0.05 (Scheffé’s test). There were significant differences between the fresh sperm group and other frozen sperm groups (not indicated). For the corresponding ANOVA analysis data, see Table 1. Mean ± SEM, n=5–7 per group.

Fig. 3. The rates of fertilization in vitro using spermatozoa frozen and thawed in cryotubes under the optimized conditions. a: The fertilization rates of spermatozoa frozen and thawed in cryotubes were significantly lower than those with fresh spermatozoa but did not differ from those of spermatozoa frozen in plastic straws (*a,a*′P<0.05). The strain had a significant effect on the fertilization rate (ICR > C57BL/6 J, P<0.05). Mean ± SEM, n=4–8 per group. b: The effect of GSH in the IVF medium on fertilization rates in the C57BL/6 J strain. The fertilization rates of spermatozoa frozen in both straws and cryotubes increased significantly with addition of GSH (P<0.05). For the corresponding ANOVA analysis data, see Table 2. Mean ± SEM, n=4 per group.

Fig. 4. The cooling rates of the cryopreservation medium in conical-bottom cryotubes at different positions from the surface of LN2. The cooling rates may vary with the position of the cryotubes. The round-bottom cryotube and the straw were placed at the –1 cm and 3–5 cm positions, respectively. The cooling rate with the round-bottom cryotube was the highest, while the rate with the straw was moderate. Mean value of two replicated experiments.
sperm cryopreservation using cryotubes have been developed, but with limited success, especially for B6J spermatozoa [23, 24]. In this study, we found that the freezing step was more critical than the thawing step for the successful survival of spermatozoa cryopreserved in cryotubes. By varying the position of the cryotube relative to the surface of the LN₂ and the volume of sperm suspension, we found that the survival of spermatozoa in cryotubes was improved to a level similar to that observed in spermatozoa frozen in plastic straws. Previous studies on the cryopreservation of mouse spermatozoa have reported optimal cooling rates of 27–130 [29] or 39–114 C/min [30]. These data are consistent with ours because the cooling rate in our experiments was –143.4 C/min in cryotubes, although there was a shoulder at around –10 C (Fig. 4). The shape of the cryotube may also affect the rate of the temperature change inside the tube during cooling and warming. We observed a faster cooling rate in round-bottom cryotubes than in conical-bottom cryotubes (–594.2 C/min and –143.4 C/min, respectively) (Fig. 4). It is very probable that our protocol is best suited for conical-bottom cryotubes and that another protocol needs to be optimized for round-bottom cryotubes.

We could confirm that the fertilization rate using the spermatozoa frozen in cryotubes was within a practical range, although addition of GSH to the fertilization medium was necessary for the B6 strain. Thus, our sperm cryopreservation protocol using conventional cryotubes may be used in laboratories that do not specialize in sperm cryopreservation. Our center has recently started receiving spermatozoa from different strains of mice for deposition, especially from overseas. Spermatozoa can be frozen at the sender’s laboratory using our protocol and sent to us safely within cryotubes.

**Methods**

Chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless stated otherwise. Epididymal spermatozoa were collected from mature ICR (Jcl:ICR, CLEA Japan, Tokyo, Japan) and B6 (C57BL/6Jcl, CLEA Japan) males aged 70–128 days, and sperm suspensions were prepared as described previously [25, 26]. In brief, the epididymides were incised with fine scissors, and the spermatozoa were allowed to disperse in 100 μl sperm freezing medium comprising 18% raffinose (cat. #217410; BD Difco, Voigt Global Distribution LLC, Kansas City, MO, USA) and 3% dehydrated skim milk (BD Difco) [20]. The sperm samples were placed in 0.25 ml plastic straws (Cassou straw; IMV Technologies, L’Aigle Cedex, France) or cryotubes (no. 366656, Nalge Nunc International, Rochester, NY, USA) in the freezing experiments and stored in LN₂ for one day or longer.

To thaw the spermatozoa, the straws or cryotubes were warmed using a water bath and an electrically controlled heating block (CFT-101, Iwaki, Asahi Glass, Tokyo, Japan) following a 1-sec and 30-sec interval at room temperature, respectively.

The cooling and warming rates were calculated for temperature ranges between 10 C and –70 C and between –70 C and –15 C, respectively. The temperature was measured twice for each condition every 3 sec using dummy straws and cryotubes containing the freezing solution inserted into a thermoprobe (Ø1 mm: BS11E-005-TS1-ASP) attached to a Thermo Printer (AP-310, Anritsu Meter, Tokyo, Japan). To assess sperm motility, sperm samples were loaded onto a microslide (0.1 × 2.0 mm, cat. #HTR499; VitroCom, Mountain Lakes, NJ, USA). The sperm motility parameters, motile percentage, progressive percentage (≥50 mm/s) and track speed, were measured in samples containing >300 sperm using an IVOS TOX automated system (Hamilton Thorne, Beverly, MA, USA). In vitro fertilization was performed using a method reported previously [17, 21]. In some experiments, 1.0 mM GSH was added to the fertilization (insemination) medium because this chemical was reported to enhance the fertilizability of oocytes [27]. In the original paper by Bath [27], GSH at higher concentrations (1.25 or 1.50 mM) was added to the fertilization medium, but under our experimental conditions, this resulted in softening of the zona pellucida, which might have hampered safe handling of fertilized oocytes. We found that 1.0 mM had a profound effect on the fertilization rates while the zona pellucida remained intact.

The fertilization rates were assessed by acetic orcein staining 4 h after sperm insemination. Some embryos that reached the two-cell stage at 24 h were transferred into the oviducts of day 0.5 pseudopregnant females (day 0 corresponds to the time of sterile mating with vasectomized males). On day 19.5, the recipient females were examined for live pups by Caesarian section.

For statistical analysis, each experiment was repeated using at least five males, and the data were analyzed by two-way ANOVA. The Scheffé’s test was used for post hoc multiple comparisons. P values <0.05 were considered significant.
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