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Cryopreservation of mouse spermatozoa

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Abstract. Recently, it has become possible to freeze a large number of mouse spermatozoa immediately after collection from the epididymides of a small number of males. The cryopreservation of spermatozoa is simpler, less time-consuming, and less costly than that of embryos for maintaining various strains of mice with induced mutations. This chapter attempts to provide a realistic overview of the cryopreservation of mouse spermatozoa and to describe a detailed procedure for mouse sperm freezing.

Introduction

Recently, a large number of various strains of mice with induced mutations (i.e., transgenes, targeted mutations, chemically induced mutations) have been produced in a variety of laboratories world wide (Jaenisch 1988; Bedell et al. 1997; Simpson et al. 1997; Hrabe de Angelis and Balling 1998; Brown and Nolan 1998). As a result, the number of strains of mice with induced mutations is rapidly expanding, and the maintenance of these strains by standard breeding colonies is becoming an increasing problem. Embryo freezing is generally used for this purpose (Pomeroy 1991). However, in the conventional method of embryo freezing, 500 embryos per strain are required from the oviducts of 20–50 mated females or by in vitro fertilization before the freezing procedure (Mobraaten 1981). In contrast, 10,000,000–30,000,000 spermatozoa can be frozen immediately after collection from the epididymides of each male. If all frozen-thawed spermatozoa from one male are used for in vitro fertilization, they can fertilize at least 500 oocytes (Nakagata 1996). Therefore, sperm freezing may provide a much simpler and economical alternative to embryo freezing to achieve this goal.

The first reports of successful cryopreservation of mouse spermatozoa were published in 1990 by three independent groups of Japanese investigators (Okuyama et al. 1990; Tada et al. 1990; Yokoyama et al. 1990). We are also subsequently successful in the cryopreservation of mouse spermatozoa by using an improved method (Takeshima et al. 1991; Nakagata and Takeshima 1992). In addition, we demonstrated for the first time that cryopreserved spermatozoa can fertilize cryopreserved oocytes in vitro and that two-cell embryos obtained from cryopreserved gametes can develop into normal live offspring after embryo transfer (Nakagata 1993).

Since that time, numerous researchers have reported successful mouse sperm cryopreservation by using various procedures (Penfold and Moore 1993; Tao et al. 1995; Sztein et al. 1997; Songsasen and Leibo 1997a, 1997b; Songsasen et al. 1997; Storey et al. 1998). Despite the many different published methods for cryopreservation, the in vitro fertilization rates and rate of development to fetus and live offspring after transfer of embryos derived from frozen spermatozoa varies considerably among different research groups. Moreover, these rates also vary with the strains of frozen sperm used and the eggs used for in vitro fertilization (Table 1). However, a comparison of these reports is not possible owing to the many differences in freezing and thawing and subsequent in vitro fertilization methods used. These findings clearly indicate that the underlying critical factors have not yet been elucidated. Therefore, development of more reliable and effective methods still appears to be required.

Our group has published numerous papers in which the basic 18% raffinose, 3% skim milk cryopreservation technique has been refined and applied to variety of different strains and transgenic stocks of mice (Nakagata and Takeshima 1993; Nakagata et al. 1995, 1997; Nakagata 1996; Okamoto et al. 1998). In addition, large-scale ENU mutagenesis screening studies have employed our cryopreservation technique to assess sperm cryopreservation for mouse mutant archiving and the rapid re-establishment of shelf stocks (Marschall et al. 1999; Thornton et al. 1999).

In this chapter, I shall restrict myself to a description of the detailed procedure routinely used for mouse sperm freezing and the reproductive technologies concerned with the sperm freezing in my laboratory.

Cryopreservation of spermatozoa

Materials and equipment

1. Male mice (3–6 months old)
2. 50-ml disposable conical tube
3. 20-ml disposable syringe, 18-gauge needle
4. Water bath
5. 2-ml sample tube
6. High-speed microcentrifuge
7. Disposable filter unit (pore size 0.45 μm; Millipore Ltd., Cat. No. SLHA025OS)
8. 1-ml glass ampules
9. Twin jet ampule sealer
10. Four-well disposable multidish (no. 176740; Nunc, Roskilde, Denmark)
11. Micropipettes
12. Tip (0.5–10 μl, 10–100 μl volume)
13. Micro-spring scissors (5-mm blade)
14. Pair of watchmakers #5 forceps
15. 35-mm sterile plastic tissue culture dishes
16. 1-ml disposable syringe
17. Straw connector (2-ml long silicone tube that fits the straw)
18. 0.25-ml insemination straw (no. A-201; IMV, I'Aigle, France)
19. Cellophane tape (12 mm wide)
20. Labels to print mouse number (5 mm × 20 mm)
21. HTF medium (Quinn et al. 1985), sterile, pregassed, plus 4 mg/ml BSA (Takahashi et al. 1995) (Table 2)
22. Impulse sealer
23. Acrylic bar (5 mm × 5 mm × 50 cm)
24. Styrofoam (30 mm thick)
25. 50-ml disposable syringe
26. Cryobiological container
27. Humidified 37°C incubator, 5% CO_2, 95% air

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Table 1. Results of frozen mouse spermatozoa.

Strain of frozen sperm	Strain of eggs	Fertilization rate (%)	Fetus (%)	Live young (%)	Reference
Inbred					
BALB/c	BALB/c	19	—[b]	—[b]	Tada et al. 1990
	B6C3F1 (C57BL/6N × C3H/HeN)	39	—[c]	—[c]	Tada et al. 1990
	ICR	48	—	35	Nakagata and Takeshima 1993
	B6C3F1(C57BL/6N × C3H/HeN)	71	52	—	Tada et al. 1993
	3H1(C3H/HeH × 101/H)	7		18	Thornton et al. 1999
C3H/HeH	3H1(C3H/HeH × 101/H)	12	—	38	Thornton et al. 1999
C3H/HeN	C3H/HeN	35	—[b]	—[b]	Tada et al. 1990
	B6C3F1(C57BL/6N × C3H/HeN)	36	—[c]	—[c]	Tada et al. 1990
	ICR	73	—	51	Nakagata and Takeshima 1993
	B6C3F1(C57BL/6N × C3H/HeN	76	68		Tada et al. 1993
C3HeB/FeJ	C3HeB/FeJ	50	—	19	Marschall et al. 1999
C57BL/6J	B6C3F1	3	—[c]	—[c]	Songsasen and Leibo 1997b
	C57BL/6J	73–85[a]	—	31–40	Nakagata et al. 1997
	3H1(C3H/HeH × 101/H)	2–20	—	0	Thornton et al. 1999
C57BL/6N	C57BL/6N	13	—[b]	—[b]	Tada et al. 1990
	B6C3F1(C57BL/6N × C3H/HeN)	35	—[c]	—[c]	Tada et al. 1990
	ICR	26	—	35	Nakagata and Takeshima 1993
	B6C3F1(C57BL/6N × C3H/HeN)	53	48	—	Tada et al. 1993
CBA/JN	ICR	77	—	48	Nakagata and Takeshima 1993
			—		
CBA/CaBlk	B6CBAF1(C57Blk/6 × CBA/CA)	50	16	17	Penfold and Moore 1993
DBA/2N	B6C3F1(C57BL/6N × C3H/HeN)	63	—[c]	—[c]	Tada et al. 1990
	DBA/2N	64	—[b]	—[b]	Tada et al. 1990
	ICR	89	—	62	Nakagata and Takeshima 1993
ddy	ddy	48	—[b]	—[b]	Tada et al. 1990
	B6C3F1(C57BL/6N × C3H/HeN)	42	—[c]	—[c]	Tada et al. 1990
kk	kk	32	—[b]	—[b]	Tada et al. 1990
	B6C3F1(C57BL/6N × C3H/HeN)	41	—[c]	—[c]	Tada et al. 1990
SAM-P/6	B6C3F1(C57BL/6N × C3H/HeN)	58	41	—	Tada et al. 1993
129/J	B6C3F1	17	—	33	Songsasen and Leibo 1997b
Closed colony					
ICR	B6C3F1(C57BL/6N × C3H/HeN)	35	—[c]	—[c]	Tada et al. 1990
	ICR	36	—[b]	—[b]	Tada et al. 1990
	ICR	42	—[c]	—[c]	Okuyama et al. 1990
	ICR	34–36	—	45	Takeshima et al. 1991
	ICR	71–95	—	51	Nakagata and Takeshima 1992
	B6C3F1(C57BL/6N × C3H/HeN)	85	57	—	Tada et al. 1993
ICR(CD-1)	ICR(CD-1)	33–58	—[c]	—[c]	Storey et al. 1998
Hybrid					
B6CF1(C57BL/6 × BALB/c)	B6CF1(C57BL/6 × BALB/c)	19–37	—	17–75	Yokoyama et al. 1990
B6C3F1(C57BL/6N × C3H/He)	ICR	59	—	56	Nakagata and Takeshima 1993
B6D2F1(C57BL/6J × DBA/2J)	CB6F1(BALB/cJ × C57BL/6J)	89–93	37	38	Ssztein et al. 1997
	B6C3F1	68	—[c]	—[c]	Songsasen and Leibo 1997a
	B6C3F1	61	—	43	Songsasen and Leibo 1997b
	B6C3F1	26–39	—	24–47	Songsasen et al. 1997
	B6C3F1	59–83		29–41	Songsasen and Leibo 1998
B6D2F1(C57BL/6N × DBA/2N)	ICR	75	—	55	Nakagata and Takeshima 1993
CD2F1(BALB/c × DBA/2N)	ICR	76	—	55	Nakagata and Takeshima 1993
C3CF1(C3H/HeH × BALB/c)	3H1(C3H/HeH × 101/H)	18–57	—	36–81	Thornton et al. 1999
Tg					
C57BL/6J	C57BL/6J	73–76[a]	—	30–31	Nakagata et al. 1997
B6ICRF1(C57BL/6N × ICR)	ICR	53	—	60	Nakagata 1996
KO					
129ICRF1(129/SvJ × ICR)	ICR	71–77	—	32–65	Okamoto et al. 1998
ENU induced mutations					
C3HeB/FeJ	C3HeB/FeJ	53	—	24	Marschall et al. 1999
			—		
C3CF1(C3H/HeH × BALB/c)	C3H/HeH	18	—	40	Thornton et al. 1999
Wild mice					
M.m. castaneus	ICR	70	—	44	Nakagata et al. 1995
M.m. domesticus	ICR	52[a]	—	17	Nakagata et al. 1995
M.m. molossinus	ICR	63–64[a]	—	20–25	Nakagata et al. 1995
M.m. musculus	ICR	48–63	—	24–51	Nakagata et al. 1995
M. spretus	3H1(C3H/HeH × 101/H)	10	—	21	Thornton et al. 1999

[a] PZD oocytes were inseminated with frozen-thawed spermatozoa with low motility.
[b] Fertilized eggs were not cultured and not transferred.
[c] Fertilized eggs were cultured to two-cell or blastocyst stage, but not transferred.

Table 2. HTF medium (Quinn 1985).

Component	mg/100 ml
NaCl	593.8
KCl	35.0
$MgSO_4 \cdot 7H_2O$	4.9
KH_2PO_4	5.4
$CaCl_2 \cdot 2H_2O$	29.8
$NaHCO_3$	210.0
Glucose	50.0
Na-pyruvate	3.7
Na-lactate[a]	0.34 ml
Penicillin G	100 U/ml
Streptomycin	50 μg/ml
BSA[b]	4 mg/ml
0.5% Phenol red	0.04 ml

[a] Assay: 70%.
[b] Highly pure BSA (Yagai Co. LTD., Yamagata, Japan, Ca. No. YH10006).

Table 3. Cryopreservation solution.

Component	g/20 ml
Raffinose	3.6
Skim milk	0.6

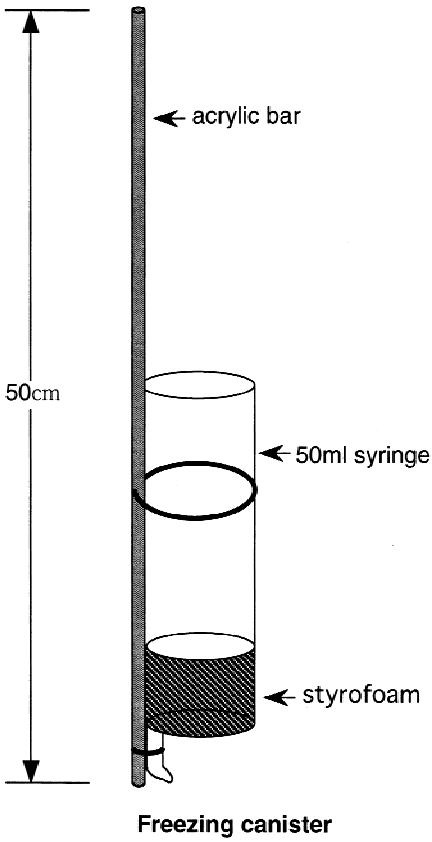

Fig. 1. Preparation of freezing canister.

Cryopreservation solution

1. Dissolve raffinose and skim milk in 20 ml of distilled water at 60°C (Table 3).
2. Draw solution into 20-ml syringe and put 1.5 ml of the solution into each 2-ml sample tube.
3. Centrifuge sample tubes at 10,000*g* for 15 min at room temperature in high-speed microcentrifuge. Comment: If the supernatant is not clear after centrifugation, centrifuge again until the supernatant becomes clear.
4. Filter supernatant with disposable filter unit and use as the cryopreservation solution (CPS).
5. Put 0.5 ml of the CPS into each 1-ml glass ampule and seal with a twin jet ampule sealer.
6. Store the CPS in each 1-ml ampule at room temperature.

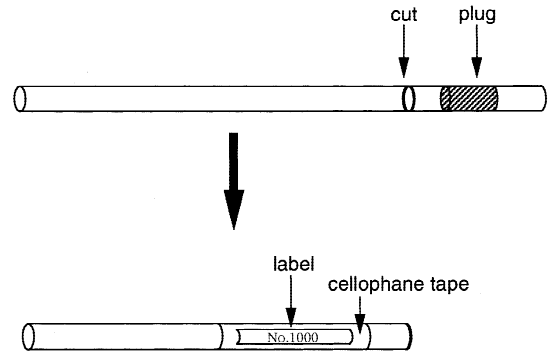

Fig. 2. Preparation of sample container.

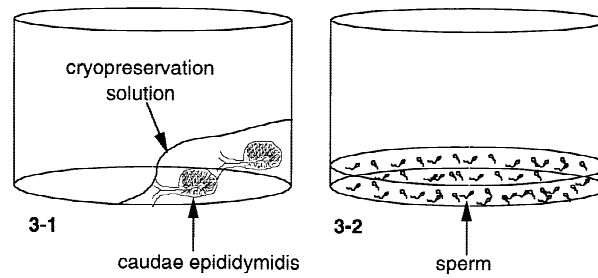

Fig. 3. Preparation of sperm suspension.

Preparation of freezing canister (Fig. 1)

1. Insert a piece of styrofoam tightly into the bottom of the syringe.
2. Heat seal outlet of syringe tip.
3. Fix the syringe to the acrylic bar (50 cm).

Preparation of sample container (Fig. 2)

1. Cut off the plug portion of the straw.
2. Apply a label with mouse number printed on it, using cellophane tape.
3. Prepare ten straws per mouse in same manner.

Procedure

Freezing:

1. Sacrifice male mouse by cervical dislocation and remove two caudae epididymides aseptically.
2. Place the two caudae epididymides into 100 μl of CPS in one well of a four-well multidish (Fig. 3-1).
3. Using a pair of watchmakers #5 forceps and micro-spring scissors, mince the epididymides and disperse spermatozoa in CPS by shaking the dish for about 2 min (Fig. 3-2).
4. Connect a 1-ml syringe and a straw using a silicone tube. Carefully aspirate 100 μl HTF medium, 10 mm air, 10 μl sperm suspension, 10 mm air, successively using a syringe into the straw, and then seal both sides of the straw with an impulse sealer (Fig. 4). Comment: The reason for loading 100 μl of

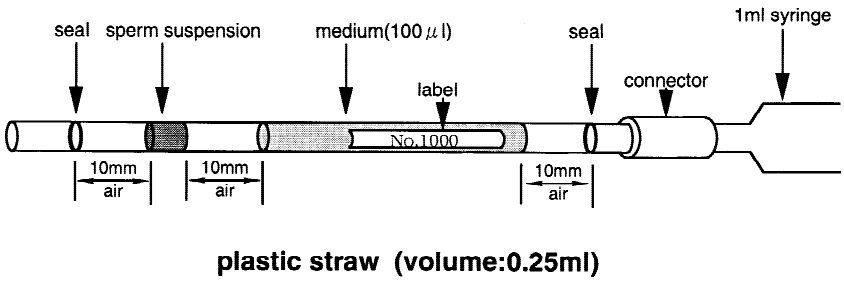

Fig. 4. Configuration of straw.

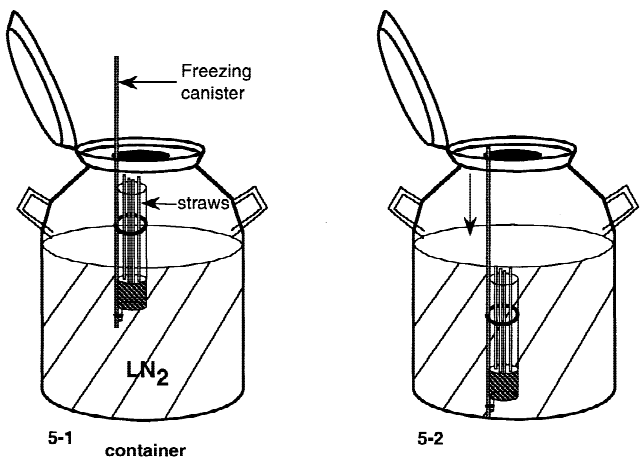

Fig. 5. Cooling of samples.

HTF medium into the straw is to prevent the straw from floating on the surface of the liquid nitrogen and enabling it to sink into the liquid nitrogen (in other words, the HTF medium acts as a weight).
5. Make up ten samples in the same manner.
6. Put the samples into a freezing canister and float on liquid nitrogen in a cryobiological container (Fig. 5-1).
7. After 10 min, sink the freezing canister into the liquid nitrogen (Fig. 5-2).

Thawing:

1. Immerse the frozen straw in a water bath maintained at 37°C (Fig. 6).
2. 15 min after immersion, remove the straw from the water bath.
3. Wipe water off straw with fine tissue paper and cut both ends of the straw.
4. Transfer only the thawed sperm suspension in the plastic dish (Fig. 7-1) and add 1–2 μl of the thawed sperm suspension to a drop of fertilizing HTF medium (200 μl) (Fig. 7-2~3).
5. Place the drops in an incubator for 1.5 h.

In vitro fertilization by using cryopreserved spermatozoa

1. Female mice superovulated with eCG and hCG
2. Paraffin oil
3. HTF medium, sterile, pregassed, plus 4 mg/ml BSA
4. Humidified 37°C incubator, 5% CO_2, 95% air
5. 35 mm sterile plastic tissue culture dishes

Procedure:

1. Sacrifice female mice 15–17 h after hCG injection and remove the oviducts.

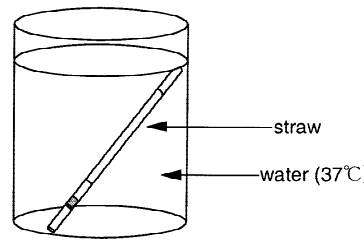

Fig. 6. Thawing of sample.

2. Using fine and sharp needles, release up to 4–6 cumulus masses into each drop of fertilizing HTF medium containing the cryopreserved spermatozoa (insemination).
3. Incubate the oocytes and spermatozoa for 4 h in an incubator.
4. Wash the oocytes twice in fresh HTF medium after incubation to prevent the harmful effects of CPS on oocytes during culture, and then culture for an additional 24 h.

Embryo transfer. In our laboratory, we usually transfer two-cell embryos to the ampulla of the recipient of pseudopregnancy through the wall of the oviduct (Nakagata 1992). This procedure is much easier than the conventional procedure of embryo transfer (Hogan et al. 1994) and is suitable for inexperienced users.

Partial zona dissection (PZD). In the case of cryopreserved spermatozoa with low fertilizing ability, we inseminate the spermatozoa with oocytes subjected to partial dissection of the zona pellucida (PZD; Nakagata et al. 1997). PZD is a fast, relatively simple procedure, and a high fertilization rate without an increase in polyspermy is obtained. Therefore, this technique is recommended for in vitro assisted fertilization.

Concluding remarks. In general, high fertilization rates are obtained for frozen spermatozoa of the CBA/JN and DBA/2N inbred strains and some F_1 hybid strains (Table 1). On the other hand, the fertilization rate of C57BL/6 frozen spermatozoa remains very low, although this can be increased by in vitro fertilization with PZD oocytes (Table 1). C57BL/6 is a major inbred strain and its genetic background is well known. Furthermore, this strain is used not only for the production of transgenic mice (Hogan et al. 1994), but is also applied as a back-cross for targeted mutant mice. Therefore, it is necessary to establish a cryopreservation method for C57BL/6 mouse spermatozoa that can maintain high fertilizing ability after thawing.

Over the past 10 years, a large number of transgenic and targeted mutant mice have been produced world wide. In addition, ENU mutagenesis projects have been progressing, leading to an enormous increase in the number of strains of mutant mice that will be produced over the next few years (Hrabe de Angelis and Balling 1998; Brown and Nolan 1998). Recently, our group has succeeded in transporting frozen spermatozoa of transgenic and

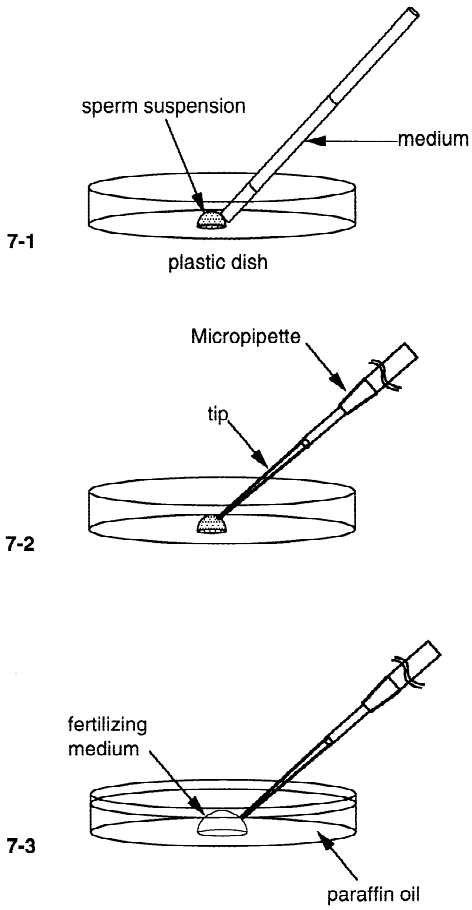

Fig. 7. Introduction of thawed spermatozoa to fertilize medium.

targeted mutant mice from USA (Jackson Lab, in Bar Harbor), England (MRC, in Harwell), and Germany (GSF, in Munich) to Japan and obtaining many live young from these transported spermatozoa (unpublished data). I believe strongly that sperm freezing represents an extremely powerful tool for storing a large number of mice with induced mutations and will see further application in the exchange of mutant strains between labs world wide.

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