—Review—

Mouse Spermatozoa Cryopreservation

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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 worldwide [1–5]. As a result, the number of strains of mice with induced mutations is rapidly increasing, and the maintenance of these strains in standard breeding colonies is becoming an increasing problem. Embryo freezing is generally used for this purpose [6]. 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 [7]. 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 [8]. Sperm freezing may therefore 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 3 independent groups of Japanese investigators [9–11]. We were also subsequently successful in the cryopreservation of mouse spermatozoa by means of an improved method [12, 13]. 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 [14].

Since that time, numerous researchers have reported successful mouse sperm cryopreservation by means of various procedures [15–26]. Nevertheless, 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 the different research groups (Table 1).

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In this paper I shall restrict myself to a description of the detailed procedure used routinely for mouse sperm freezing and the reproduction methods related to sperm freezing in my laboratory.

1 Cryopreservation of spermatozoa

1-1 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 4-well disposable multi-dish (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, l'Aigle, France)
19 Cellophane tape (12 mm wide)
20 Labels to print mouse number (5 mm $\times$ 20 mm)
21 HTF medium [27], sterile, pregassed, plus 4 mg/mL BSA [28] (Table 2)
22 Impulse sealer
23 Acrylic bar (5 mm $\times$ 5 mm $\times$ 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

Table 1. Results for frozen mouse spermatozoa

Strain	Reference	Fertilization rate (%)	Fetus (%)	Live young (%)
<Inbred>				
BALB/c	Tada *et al.* [10]	19–39	-**	-**
	Nakagata & Takeshima [15]	48	-	35
	Tada *et al.* [16]	71	52	-
C3H/He	Tada *et al.* [10]	35–36	-**	-**
	Nakagata & Takeshima [15]	73	-	51
	Tada *et al.* [16]	76	68	-
C57BL/6N	Tada *et al.* [10]	13–35	19	-
	Nakagata & Takeshima [15]	26	-	35
	Tada *et al.* [16]	53	48	-
C57BL/6J	Songsasen and Leibo [22]	0	-**	-**
	Nakagata *et al.* [31]	73–85*	-	31–40
CBA/2N	Nakagata & Takeshima [15]	77	-	48
CBA/CaBlk	Penfold & Moore [17]	50	16	17
DBA/2N	Tada *et al.* [10]	63–64	-**	-**
	Nakagata & Takeshima [15]	89	-	62
ddy	Tada *et al.* [10]	42–48	-**	-**
kk	Tada *et al.* [10]	32–41	-**	-**
SAM-P/6	Tada *et al.* [16]	58	41	-
129/SvJ	Songsasen and Leibo [22]	13	-	31
<Closed colony>				
ICR	Tada *et al.* [10]	35–36	-**	-**
	Okuyama *et al.* [9]	42	-**	-**
	Takeshima *et al.* [12]	34–36	-	45
	Nakagata & Takeshima [15]	71–95	-	51
	Tada *et al.* [16]	85	57	-
ICR (CD-1)	Storey *et al.* [25]	33–58	-**	-**
<hybrid>				
B6CF1 (C57BL/6 X BALB/c)	Yokoyama *et al.* [11]	19–37	-	17–75
B6C3F1 (C57BL/6N X C3H/He)	Nakagata & Takeshima [15]	59	-	56
B6D2F1 (C57BL/6J X DBA/2J)	Sztein *et al.* [20]	89–93	37	38
	Songsasen and Leibo [21]	64–73	-**	-**
	Songsasen and Leibo [22]	61	-	42
	Songsasen *et al.* [23]	26–39	-	24–47
BDF1 (C57BL/6N X DBA/2N)	Nakagata & Takeshima [15]	75	-	55
CDF1(BALB/c X DBA/2N)	Nakagata & Takeshima [15]	76	-	55
<Tg>				
C57BL/6J	Nakagata *et al.* [31]	73–76*	-	30–31
B6 (B6ICRF1)	Nakagata *et al.* [8]	53	-	60
<KO>				
129ICRF1	Okamoto *et al.* [26]	71–77	-	32–65
<Wild mice>				
M.m.castaneus	Nakagata *et al.* [18]	70	-	44
M.m.domesticus	Nakagata *et al.* [18]	52*	-	17
M.m.molossinus	Nakagata *et al.* [18]	63–64*	-	20–25
M.m.musculus	Nakagata *et al.* [18]	48–63	-	24–51

*PZD oocytes were inseminated with frozen-thawed spermatozoa with low motility. **Fertilized oocytes were not transferred.

Table 2. HTF medium

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[1)]	0.34 ml
Penicillin G	100 U/ml
Streptomycin	50 μg/ml
BSA[2)]	4 mg/ml
0.5% Phenol red	0.04 ml

1) Assay; 70%. 2) 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

1-2 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 centrifuging, centrifuge again until the supernatant becomes clear.
4 Filter supernatant through disposable filter unit and use as the cryopreservation solution (CPS).
5 Put 0.5 mL of the CPS into individual 1-mL glass ampules and seal with a twin jet ampule sealer.
6 Store the CPS in individual 1-mL ampules at room temperature.

1-3 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).

1-4 Preparation of sample container (Fig. 2)

1 Cut off the plug portion of the straw.

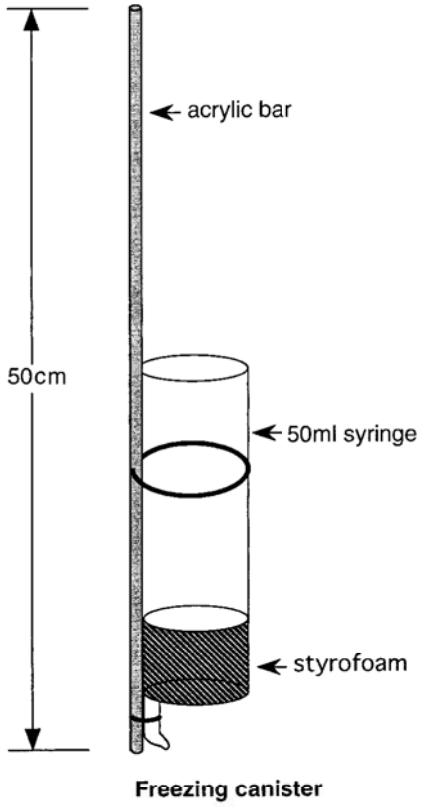

Fig. 1. Preparation of freezing canister

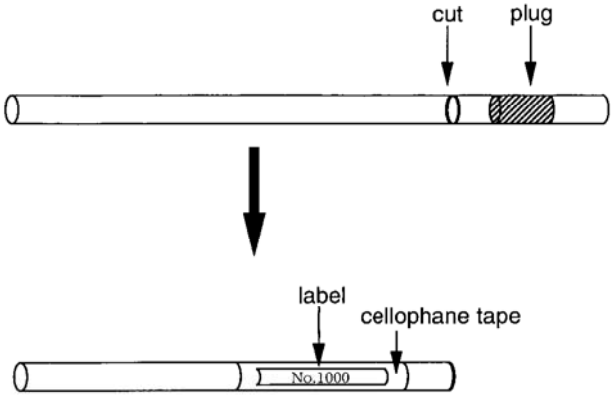

Fig. 2. Preparation of sample container

2 Apply a label with mouse number printed on it with cellophane tape.
3 Prepare ten straws per mouse in the same manner.

1-5 Procedure

1-5-1 Freezing

1 Sacrifice male mouse by cervical dislocation and

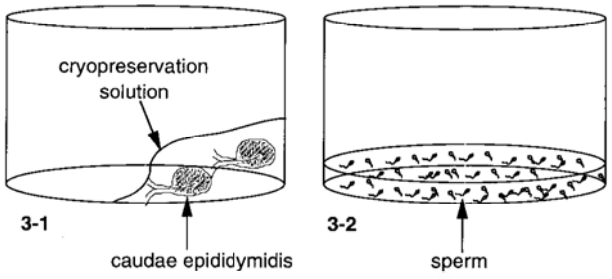

Fig. 3. Preparation of sperm suspension

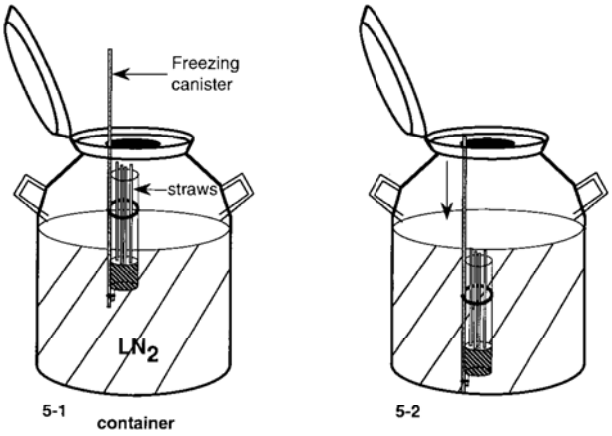

Fig. 5. Cooling of samples

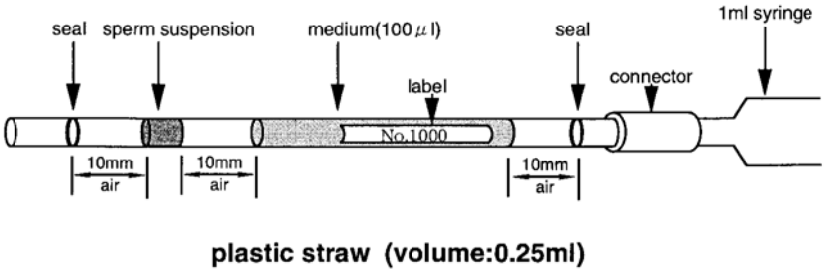

Fig. 4. Configuration of straw

remove two caudae epididymides aseptically.

2 Place the two caudae epididymides into 100 μL of CPS in each well of a 4-well multi dish (Fig. 3-1).

3 With 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 by means of a silicone tube. Carefully aspirate 100 μL HTF medium, 10 mm air, 10 μL sperm suspension, 10 mm air, successively with a syringe inserted into the straw and then seal both ends of the straw with an impulse sealer (Fig. 4).
Comment: The reason for loading 100 μL of HTF medium into the straw is to prevent the straw from floating on the surface of the liquid nitrogen and to enable 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).

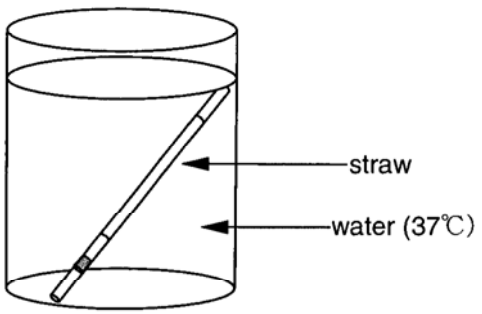

Fig. 6. Thawing of sample

7 After 10 min, sink the freezing canister into the liquid nitrogen (Fig. 5-2).

1-5-2 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 from the straw with fine tissues and cut off both ends of the straw.

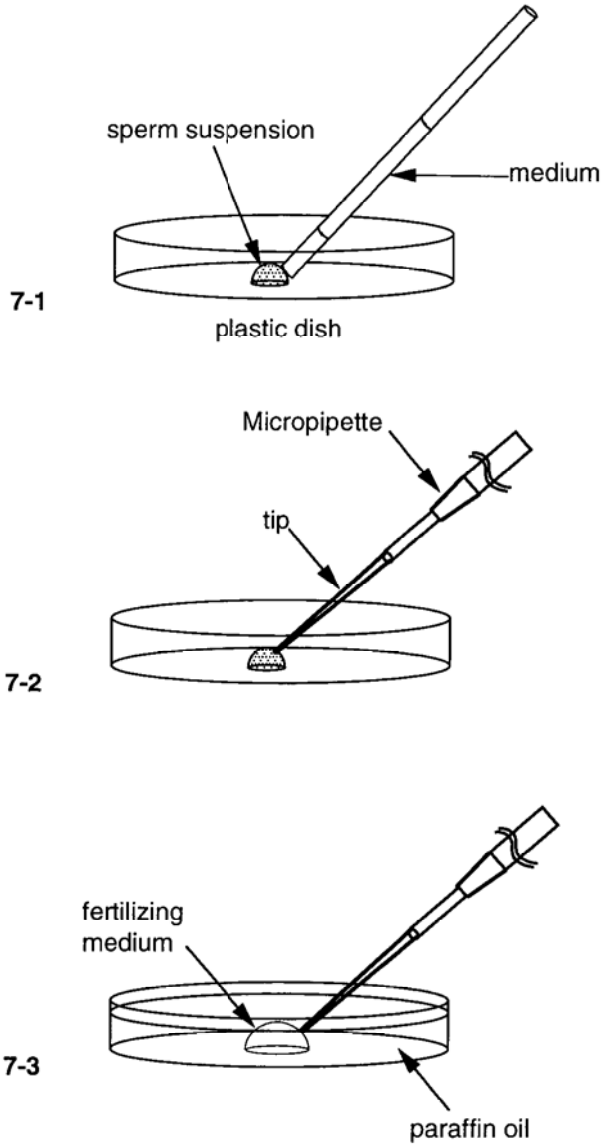

Fig. 7. Introduction of thawed spermatozoa into fertilizing medium

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.

2 *In vitro* Fertilization with Cryopreserved Spermatozoa

2-1 Materials and equipment
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

2-2 Procedure
1 Sacrifice female mice 15–17 h after hCG injection and remove the oviducts.
2 With 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.

3 Embryo Transfer

In our laboratory, we usually transfer 2-cell embryos to the ampulla of the recipient of pseudopregnancy through the wall of the oviduct [29]. This procedure is much easier than the conventional procedure for embryo transfer [30] and is suitable for inexperienced users.

3-1 Materials and equipment
1 Female mice on Day 1 of pseudopregnancy (day on which vaginal plug is observed).
2 Micro-spring scissors (5 mm blade).
3 Pair of watchmakers #5 forceps.
4 Wound clip (Autoclip 9 mm; Clay Adams 427631) and clip applicator (Mik-Ron Autoclip Applier; Clay Adams 427630).
5 35-mm sterile plastic tissue culture dishes.
6 Glass capillary (200–250 μm external diameter of tip) for embryo transfer.

3-2 Procedure
1 Anesthetize a female mouse.
2 Bring the ovary, oviduct and part of the uterine horn to the exterior according to the conventional procedure [30].
3 With a pair of watchmakers #5 forceps and micro-spring scissors, dissect the wall of the oviduct between the fimbria and ampulla.
4 Introduce the tip of the prepared capillary with 8–10 embryos and a few air bubbles from the slit into the oviduct toward the ampulla side (Fig. 8-1).
5 Expel the embryos and 2–3 air bubbles (Fig. 8-2).
6 Observe the bubbles through the wall of the am-

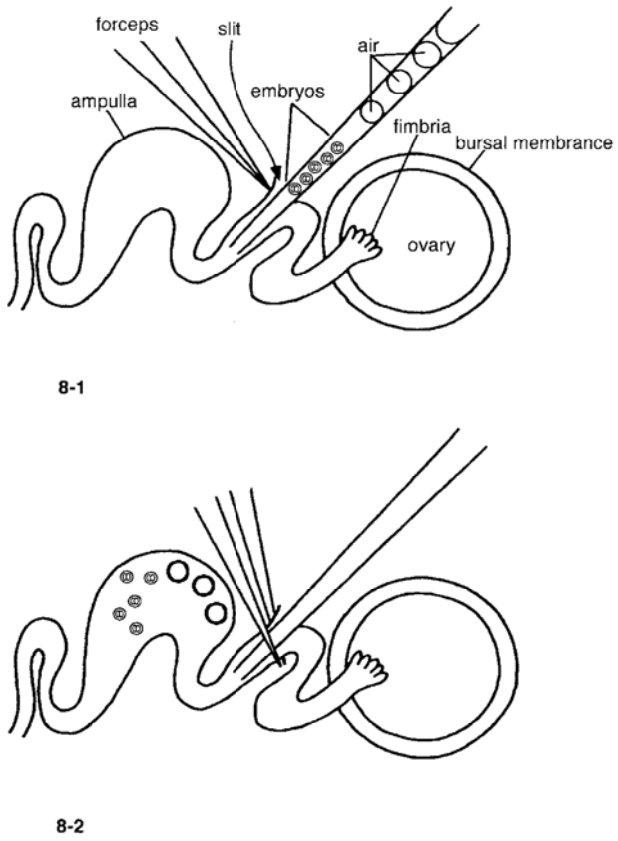

Fig. 8. Procedure for embryo transfer through the wall of the oviduct

Table 4. PB1 medium

Component	mg/100 ml
NaCl	800.0
KCl	20.0
$CaCl_2 \cdot 2H_2O$	13.2
KH_2PO4	20.0
$MgCl_2 \cdot 6H_2O$	10.0
$Na_2HPO_4 \cdot 12H_2O$	289.8
Glucose	100.0
Na-Pyruvate	3.6
Penicillin G	100 U/ml

pulla, and then gently withdraw the capillary.

7 Return the ovary and oviduct to the abdomen and close the skin with wound clips.

4 Partial Zona Dissection (PZD)

In the case of cryopreserved spermatozoa with low fertilizing ability, we use the spermatozoa to inseminate oocytes subjected to partial dissection of the zona pellucida (PZD) [31].

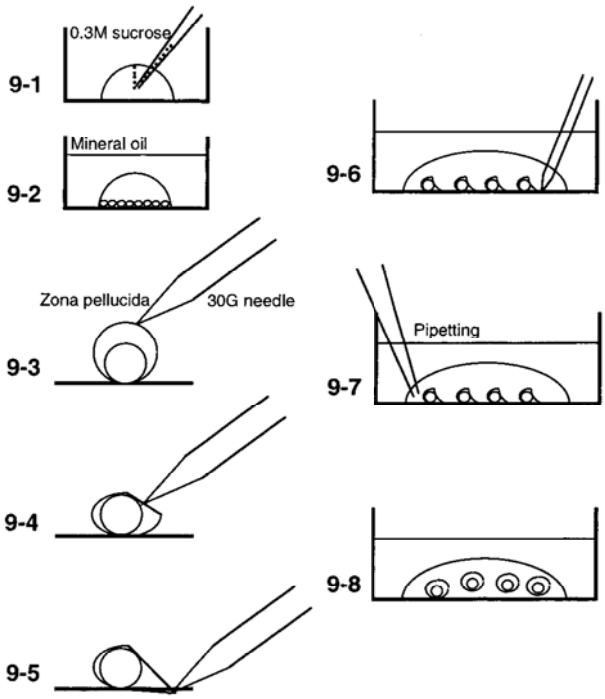

Fig. 9. PZD procedure

4-1 Materials and equipment

1 Female mice superovulated with eCG and hCG
2 HTF medium, sterile, pregassed, plus 4 mg/mL BSA
3 0.1% hyaluronidase solution in HTF solution
4 35-mm sterile plastic tissue culture dishes
5 Paraffin oil
6 0.3 M sucrose solution in PB1 medium [32] (Table 4)
7 1-mL disposable syringe, 30 gauge needle
8 0.3 M sucrose solution in PB1 medium containing BSA (40 mg/mL)
9 Micropipette
10 Tip (10–100 μL volume)

4-1 Procedure

4-2-1 PZD

1 Collect oocytes from the oviducts of superovulated females (see section 2-2) and remove cumulus cells around oocytes with 0.1% hyaluronidase solution.
2 Introduce the denuded oocytes into the top of a 100 μL drop of 0.3 M sucrose solution on a dish (Fig. 9-1).

Comments: The oocytes are at first located near the top of the solution and then descend and attach to the bottom of the dish. This attachment is caused by a charge difference between the oocytes and the dish surface (Fig. 9-2). The hypotonic solution results from the increased perivitelline space created by oocyte dehydration.

3 Partially dissect the zona pellucida of the attached oocytes by a single downward motion with a 30 gauge needle under a stereomicroscope (Fig. 9-3~6).

4 After PZD, neutralize the electrostatic attraction of the zona surface by adding of 20 μL of 0.3 M sucrose solution with BSA.

5 To remove PZD oocytes from the dish surface, spray sucrose solution onto the oocytes with a micropipette (Fig. 9-7~8).
Comment: The spray should be applied from the opposite side of the slit to prevent the escape of ooplasm from the zona pellucida.

6 Wash PZD oocytes gently 3 times in HTF medium to remove sucrose.

4-2-2 *In vitro* fertilization and embryo transfer

1 Introduce the PZD oocytes into the HTF medium containing the frozen-thawed spermatozoa prepared previously (insemination) (see section 1-5-2).

2 Wash the oocytes twice gently in the fresh HTF medium at 4 h after insemination and culture for 3 days until the fertilized oocytes are developed to the early blastocyst stage.

3 Transfer the early blastocysts into the uterine horns of a recipient on Day 3 of pseudopregnancy (Day 1 is the day on which a vaginal plug is observed).
Comment: If the oocytes are transferred to the oviducts of recipients on Day 1 of pseudopregnancy at the 2-cell stage, the rate of development into live offspring is very low.

Concluding Remarks

In general, high fertilization rates are obtained for frozen spermatozoa of the CBA/2N and DBA/2N inbred strain, and some F1 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 [30], 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 ten years, a large number of transgenic and targeted mutant mice have been produced worldwide. 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 [4, 5]. Recently our group succeeded in transporting frozen spermatozoa of transgenic and targeted mutant mice from the USA (Jackson lab, in Bar Harbor) 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 large numbers of mice with induced mutations and will see application in the exchange of mutant strains between labs worldwide.

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