Comparative Medicine
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Vol 57, No 5
October 2007
Pages 469–475

Phenotypic Analysis of C57BL/6J and FVB/NJ Mice Generated Using Evaporatively Dried Spermatozoa

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Combination of evaporative drying and frozen storage at –80 °C has been used successfully to preserve hybrid B6D2F1 mouse spermatozoa. To determine whether this method can be applied equally well to inbred mice, spermatozoa of C57BL/6J and FVB/NJ mice were evaporatively dried and stored for 1 mo at –80 °C before being used for intracytoplasmic sperm injection (ICSI) to produce live offspring. After weaning, 1 male and 1 female mouse from each litter were randomly selected at 8 wk of age for natural mating to produce live offspring. Results showed that spermatozoa from both inbred strains that had been evaporatively dried and subsequently stored at –80 °C could be used successfully to derive live, healthy, and reproductively sound offspring by ICSI. No significant differences were found in embryo transfer rate (number of pups born/number of embryos transferred), litter size, weaning rate, body weight, number of pathologic lesions, and amount of contamination by pathogens of mice produced by ICSI using evaporatively dried spermatozoa compared with mice produced by natural mating or by ICSI using fresh (that is, nonpreserved) spermatozoa. Progeny produced by mating mice generated from ICSI using evaporatively dried spermatozoa were normal. Therefore, spermatozoa from inbred mouse strains C57BL/6J and FVB/NJ can be preserved successfully after evaporative drying and frozen storage at –80 °C.

Abbreviations: BSA, bovine serum albumin; EGTA, ethylene glycol tetraacetic acid; ICSI, intracytoplasmic sperm injection

Cryopreservation of mouse sperm in liquid nitrogen was first reported in 1990.[20,24,29] Today the most widely used method for mouse sperm cryopreservation is the Nakagata protocol.[17] The purpose of sperm cryopreservation is to preserve the viability and motility of the spermatozoa so that the spermatozoa, after thawing, can be used to fertilize ova by in vitro fertilization or by artificial insemination. However, spermatozoa from some genetically inbred mouse strains (for example, C57BL/6, BALB/c, 129/Sv) exhibit substantial variability in survival, recovery, and especially motility after cryopreservation because of inherent susceptibilities to cryodamage.[8,17-21,23,25] This variability limits the success of artificial reproductive techniques, such as in vitro fertilization and artificial insemination, using cyopreserved spermatozoa, and thus hampers the use of sperm cryopreservation procedures for archiving and preserving mutant mouse lines on inbred backgrounds, especially C57BL/6.

One means by which to address infertility due to poor sperm motility after cryopreservation is to use intracytoplasmic sperm injection (ICSI), a micromanipulation process in which a spermatozoon is mechanically injected into the cytoplasm of an ovum.[10,14] Spermatozoa need not be fertile for use in ICSI, and for mice, only the sperm head is needed to successfully fertilize ova by ICSI.[22] In recent years, these features of ICSI have enabled the introduction of alternative methods for the preservation of mouse spermatozoa, including freeze drying[7,9,11,26,27] and evaporative drying.[2,13,15]

Received: 12 Mar 2007. Revision requested: 17 Apr 2007. Accepted: 30 Apr 2007.
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Evaporative drying has considerable advantages over freeze drying. For example, evaporative drying does not require the use of liquid nitrogen. In addition, evaporative drying does not require a freeze-drying machine, because water is removed from the spermatozoa by directing a stream of inert nitrogen gas at ambient temperature over the sperm sample. Thus, evaporative drying is a far simpler, faster, and cheaper method than is freeze drying.

A consideration common to both freeze drying and evaporative drying, however, is the need for very cold temperatures for storage of mouse spermatozoa dried by either method. Mouse spermatozoa that have been evaporatively dried deteriorate, and stored long-term at ambient room (25 °C) or refrigerator (4 °C) temperatures, are not reliable for producing liveborn offspring using ICSI.[7,9,13,15] In a previous study, we demonstrated that hybrid B6D2F1 mouse spermatozoa that had been evaporatively dried in trehalose–ethylene glycol tetracetic acid solution and stored at –80 °C for at least 5 mo could be used successfully for ICSI to produce normal and viable offspring.[13] The present study was designed to determine whether dried and frozen inbred, wild-type mouse spermatozoa could be used for ICSI to produce viable offspring and to assess whether those offspring were reproductively sound.

Materials and Methods

Reagents and media. KSOM^{AA} medium[3] and FHM medium[12] were purchased from Specialty Media (Phillipsburg, NJ). Polyvinyl pyrrolidone was purchased from Irvine Scientific (Santa Ana, CA), mercury from Fisher Scientific, and α-trehalose dehydrate from Ferro-Pfanstiehl Laboratories (Waukegan, IL). Analgesic (buprenorphine [Buprenex]) was purchased from Western Medical Supply (Arcadia, CA). Pregnant mare serum gonadotrophin,

human chorionic gonadotrophin and all other chemicals were obtained from Sigma Chemical (St Louis, MO). Na-K-EGTA medium used to collect spermatozoa was Tris-buffered ethylene glycol tetraacetic acid (EGTA) solution containing 10 mM Tris-HCl, 50 mM NaCl, and 50 mM EGTA, pH-adjusted to 8.2 by using KOH solution. Na-K-EGTA medium alone and supplemented with 1.0 M trehalose were stored at 4 °C after sterile filtration and used within 4 and 2 wk, respectively. Used to create transmembrane channels in the sperm plasma membrane through which trehalose was loaded, α hemolysin from *Staphylococcus aureus* (Sigma Chemical), was prepared as a 2× stock solution (25 µg/ml) in a buffered saline solution containing 10 mM NaCl, 120 mM KCl, 5 mM glucose, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and was stored at –20 °C before use.

Animals. C57BL/6J and FVB/NJ mice (female, 6 to 8 wk of age and used as egg donors; male, 10 to 12 wk of age and used as sperm donors) were purchased from The Jackson Laboratory (Bar Harbor, ME); 8- to 10-wk-old CD1 mice (Charles River Laboratories, Wilmington, MA) were used to produce vasectomized males and pseudopregnant recipients (0.5 days postcoitum) for embryo transfer. All mice were housed in individually ventilated plastic cages (BioZone, Fort Mill, SC) with bedding made from reclaimed wood pulp (Absorption Corporation, Bellingham, WA) in a specific-pathogen–free barrier facility with a 14:10-h light:dark cycle according to standard operating procedures of the Center for Laboratory Animal Science (University of California, Davis, CA). Mouse food was purchased from LabDiet (Richmond, IN), and water was deionized and autoclaved. Mouse euthanasia was carried out by CO_2 administration followed by cervical dislocation. The care, use, and disposition of all mice used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California at Davis.

Experimental design. Unfertilized ova were fertilized by using 3 types of sperm delivery: natural mating, ICSI using fresh spermatozoa, and ICSI using rehydrated evaporatively dried spermatozoa after storage for 30 d at –80 °C. For both C57BL/6J and FVB/NJ inbred mouse lines, 5 or 6 male mice were used as sires for natural mating (1 male and 1 female mouse in each breeding pair); female mice were induced to superovulate by injections of pregnant mare serum gonadotrophin and human chorionic gonadotrophin according to standard procedures (described in a following section). Fertilized 1-cell-stage eggs were collected from the oviducts of plugged females (natural mating group).

The male mouse that had been used to produce the naturally fertilized embryos was euthanized, and its spermatozoa were collected from the caudal epididymides into prewarmed Na-K-EGTA medium. The freshly harvested spermatozoa were split into 2 samples, one of which was used immediately for ICSI into freshly harvested ova to produce fertilized embryos (fresh sperm group). The other aliquot was evaporatively dried and subsequently stored for 30 d at –80 °C, after which time it was rehydrated and used for ICSI into freshly harvested ova to produce fertilized embryos (evaporated sperm group). Fertilized 1-cell stage eggs from all 3 experimental groups were cultured in KSOM^{AA} medium. After reaching the 2-cell stage, embryos were transferred into the oviducts of pseudopregnant female CD1 recipient mice 0.5 d postcoitus with a vasectomized stud male CD1 mouse confirmed to be sterile by using standard techniques.[16] All pregnant recipients were allowed to go to term and give birth (first generation).

Figure 1. Evaporative drying system for sperm. Compressed ultrapure nitrogen gas is blown through a regulator and flow meter, into the drying chamber, and out through a hole at the right end of the drying chamber. A glass slide with 2 10-mm circular areas and a silicone isolator and a sperm sample on a glass slide vacuum-sealed in a metallized nylon bag also are shown.

For each strain, the date of birth of each litter was recorded, and the body weight of each pup was measured on days 14, 28, 35, and 42. All pups were weaned at 21 d of age. When the progeny were 8 wk old, 1 male and 1 female mouse from each of 4 to 6 litters within a group were chosen randomly as breeding pairs for natural mating to produce offspring of the second generation. The pups of the second generation were weaned at 21 d of age, and body weights were measured on days 14, 28, and 42.

Sperm treatment and evaporative drying. Sperm samples were collected and evaporatively dried by using methods published previously.[13,15] Briefly, male mice were euthanized, and spermatozoa were harvested into 0.5 ml of prewarmed Na-K-EGTA medium by gently puncturing and expressing spermatozoa from the caudal epididymides by using sterile needles under a dissecting microscope. After removal of the epididymal tissue, spermatozoa were incubated at 37.5 °C for 10 min to allow for sperm dispersion. Then, 100 µl sperm suspension was mixed with an equal volume of 25 µg/ml α-hemolysin solution for 30 min at room temperature in the dark to permeabilize spermatozoa. After permeabilization, 200 µl of 1.0 M trehalose in Na-K-EGTA medium was added (final concentration of trehalose, 0.5 M) to the sperm suspension, mixed, and allowed to sit for 15 min at room temperature in the dark for trehalose loading. Sperm samples were kept on ice before evaporative drying.

Instrumentation and procedures for evaporative drying were the same as those described previously[2,13,15] (Figure 1). The drying chamber was made by the Center for Engineering in Medicine (Massachusetts General Hospital, Harvard Medical School, Boston, MA). Briefly, after trehalose loading, 20-µl aliquots of sperm suspensions were placed within a 10-mm circular area demarcated on a sterile glass slide (Ted Pella, Redding, CA). The slide then was placed into a evaporative drying chamber and evaporatively dried by blowing of compressed ultrapure-grade nitrogen gas directly through the chamber for 5 min at a flow rate of 10 l/min.

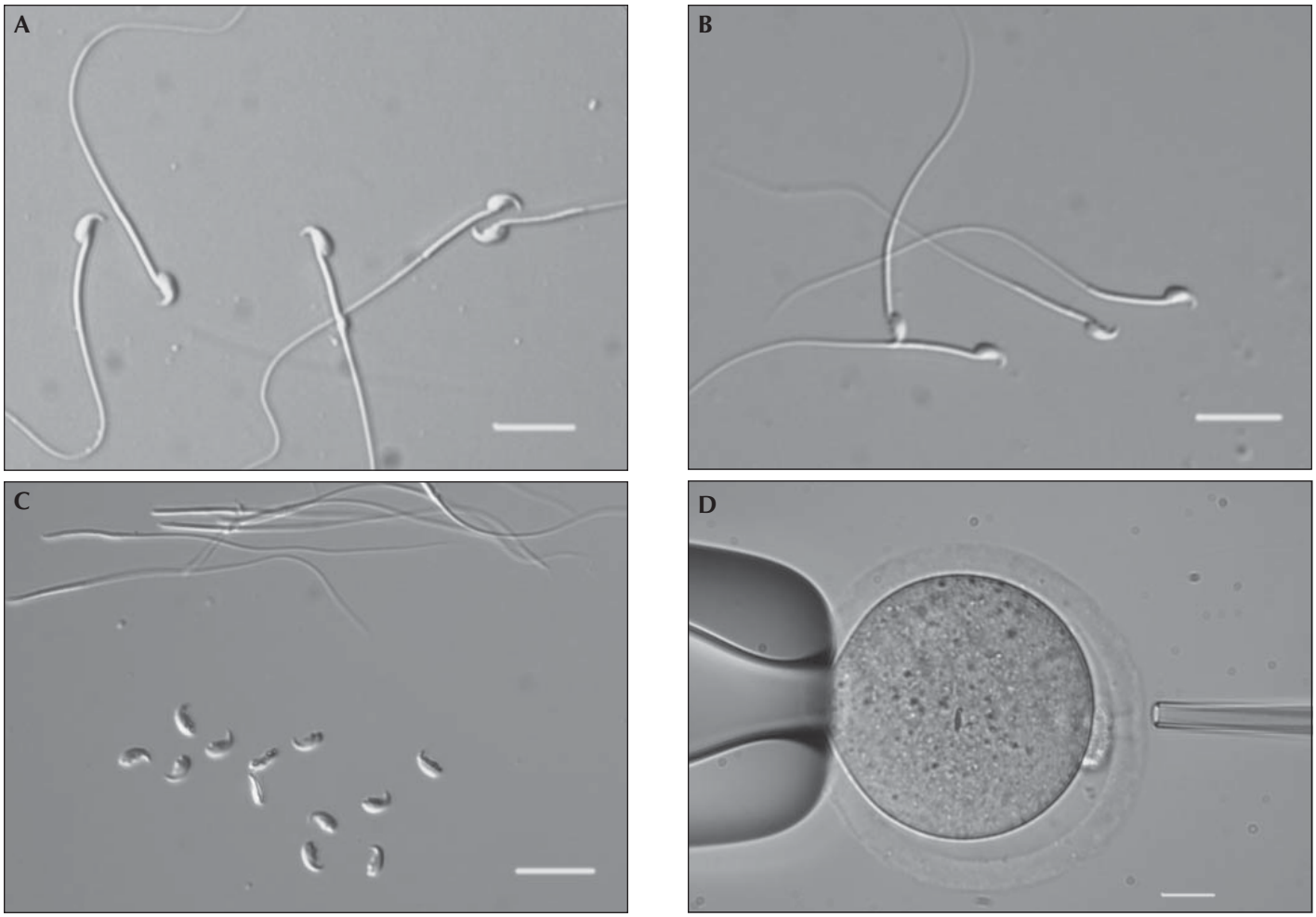

Figure 2. Photomicrographs of (A) fresh C57BL/6J sperm and (B) evaporatively dried sperm after storage at –80 °C for 10 mo, (C) sperm heads separated from sperm tails by piezoelectricity-driven needle, and (D) an ovum after ICSI of a sperm head. Fresh sperm were incubated in 10% polyvinyl pyrrolidone in FHM medium for about 1 h prior to ICSI. Bar, 20 µm in A, B, C and 15 µm in D.

Afterward, a silicone isolator (Press-to-Seal Without Adhesive, Grace Bio-Labs, Bend, OR) was placed around the partially dried sperm sample and covered with a sterile glass coverslip. The slide then was vacuum-sealed in a metallized nylon bag (Impak, Los Angeles, CA) by using a vacuum sealer (Impak) before being stored at –80 °C for 30 d with the sperm samples facing up.

Superovulation and ICSI. Female mice were superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin, followed 46 to 48 h later by intraperitoneal injection of 5 IU human chorionic gonadotrophin; mice were euthanized 14 to 15 h later after injection, and oocyte–cumulus complexes were collected from oviducts and placed into warm FHM medium. Cumulus cells were dissociated from oocyte–cumulus complexes by treatment with 300 U/ml bovine testis hyaluronidase in FHM medium for about 5 min at room temperature. Oocytes were washed in preequilibrated KSOM^{AA} medium and then cultured in this medium at 37.5 °C in humidified 5% CO_2 and 95% air before being used for ICSI. Oocytes were used for ICSI within 3 h after collection.

ICSI was performed on an inverted microscope with Narishige micromanipulators (TE200, Nikon, Melville, NY) at room temperature, as described previously.[13] Briefly, the package containing the sperm sample was thawed at 37 °C in air for 5 min before opening. The cover glass and gasket were removed, and the sperm sample was rehydrated by addition of 20 µl of FHM medium (without bovine serum albumin [BSA]) containing 0.01% (w/v) polyvinyl alcohol (cold-water–soluble; molecular weight, 30 to 70 kDa). A small amount (approximately 0.1 µl) of rehydrated sperm suspension was transferred by using a mouth pipette into a 5-µl drop of 10% polyvinyl pyrrolidone (w/v, in BSA-free FHM medium) under mineral oil and mixed thoroughly. An individual spermatozoon was aspirated, tail first, into the ICSI pipette (diameter, approximately 7 µm), and then 3 to 6 piezoelectric pulses were applied to the neck region (intensity, 4 to 5; speed, 3 to 4; PMM Controller, Prime Tech, Ibaraki, Japan) to separate the sperm head from the tail. A group of about 10 sperm heads (Figure 2) was washed in another drop of 10% polyvinyl pyrrolidone before being loaded into the ICSI pipette.

ICSI was done in a drop of BSA-free FHM medium containing 0.01% PVA. Piezoelectric parameters used for zona penetration were an intensity of 2 to 3 and speed of 3 and for oolemma breaking were an intensity of 1 to 2 and speed of 1. Injected oocytes (Figure 2) were washed, incubated in equilibrated KSOM^{AA} medium (50-µl drops under mineral oil) at 37.5 °C in humidified 5% CO_2 and 95% air, and cultured overnight. Embryos that reached the 2-cell stage were transferred into the oviducts of 0.5 d post-

Table 1. Fertilization, embryo transfer, and weaning rates and sex ratios of mice produced by natural mating, after ICSI with fresh spermatozoa, and after ICSI with evaporatively dried spermatozoa after 30 d of storage at –80 °C (first generation)

	Natural mating	Fresh sperm	Evaporated sperm
C57BL/6 mice			
No. of male mice used	6	6	5
No. of female mice used	6	7	6
No. of ova injected	not applicable	116	104
No. of ova that survived ICSI (%)	not applicable	92 (79)	87 (84)
No. of 2-cell embryos obtained[a]	101	87	80
Fertilization rate (%)[b]	not applicable	94.6	92.0
No. of recipients used	6	7	6
No. of litters obtained	6	7	6
No. of pups born	35	38	36
Embryo transfer rate[c]	34.7	43.7	45.0
Weaning rate (%)	100	100	100
Sex ratio (male/female)	1.3 (20/15)	2.8 (28/10)	2.3 (25/11)
FVB/N mice			
No. of male mice used	5	5	5
No. of female mice used	7	6	8
No. of ova injected	not applicable	133	165
No. of ova that survived ICSI (%)	not applicable	116 (87)	148 (90)
No. of 2-cell embryos obtained[a]	107	108	138
Fertilization rate (%)	not applicable	93.1	93.2
No. of recipients used	7	6	8
No. of litters obtained	7	6	8
No. of pups born	54	40	50
Embryo transfer rate	50.5	37.0	36.2
Weaning rate (%)	100	100	100
Sex ratio (male/female)	0.7 (23/31)	0.8 (18/22)	1.2 (27/23)

[a]All of the 2-cell embryos obtained were transferred.
[b]100% × no. of 2-cell embryos obtained/no. of ova that survived.
[c]100% × no. of pups born/no. of 2-cell embryos obtained.

coitus pseudopregnant CD1 recipients anesthetized with 2.5% tribromoethanol. Recipients were kept warm on a heating pad until fully recovered from anesthesia. Before the mouse was fully conscious, 0.1 ml of 0.03 mg/ml buprenorphine was injected subcutaneously to provide postoperative analgesia.

Necropsy and pathology examination. The procedures of sperm collection, sperm evaporative drying, sperm storage, and ICSI were performed in a nonspecific-pathogen–free barrier laboratory. To determine whether ICSI, evaporative drying, and storage at –80 °C damaged the spermatozoa sufficiently to produce nonlethal pathologic defects in liveborn offspring, 1 or 2, 6- to 7-wk-old mice (1 male and 1 female when available) from each of 5 representative litters from each of the 2 generations of each of the 3 experimental groups were randomly selected for gross necropsy and histologic pathology. Organs for histologic pathology included liver, kidney, spleen, mesenteric lymph node, pancreas, heart, lung, thymus, brain, adrenal gland, reproductive tract, urinary bladder, stomach, cecum, small intestine, and colon.

To ensure that any physiologic, reproductive, or pathologic abnormalities were not secondary to contamination caused by adventitious pathogens, diagnostic screening for infectious agents was performed on 1 or 2 random 6- to 7-wk-old mice from each of the 5 representative litters in the first generation from the 3 experimental groups. The screening included gross examination, cecal and nasopharyngeal aerobic cultures for bacterial pathogens, cecal and pelage examination for parasites (pinworms and fur mites), and enzyme-linked immunosorbent assay to detect serum antibodies to mouse hepatitis virus, Sendai virus, pneumonia virus of mice, mouse parvovirus, minute virus of mice, *Mycoplasma pulmonis*, Theiler murine encephalomyelitis virus, ectromelia virus, rotavirus, mouse adenovirus 1 and 2, lymphocytic choriomeningitis virus, and reovirus 3.

Statistical analysis. Data are expressed as mean ± standard error of the mean. One-way analysis of variance and the Student *t* test were applied, and the probabilities were computed exactly using StatXact 7 software (Cytel Statistical Software, Cambridge, MA). A *P* value of less than 0.05 was considered statistically significant.

Results

Effect of sperm evaporative drying and frozen storage on fertilization rate and embryo development. The fertilization rates (no. of 2-cell embryos/no. ova that survived ICSI) of ova injected with fresh spermatozoa and evaporatively dried spermatozoa followed by 30 d storage at –80 °C were similar among and between C57BL/6J and FVB/NJ mice (Table 1). In addition, the embryo transfer rates (no. of pups born/no. of 2-cell embryos obtained) by using 2-cell embryos produced after natural mating and 2-cell embryos produced by ICSI using either fresh or evaporatively dried spermatozoa were similar among mice of each strain and between the 2 strains. Further, all of the pups born to both strains of mice were weaned successfully (weaning rate 100% in all 3 experimental groups). More male pups were obtained in both the fresh sperm group and evaporatively dried sperm group of C57BL/6J mice, but the ratios were not significantly different from the ratio in the natural mating group.

For both the C57BL/6J and FVB/NJ strains, evaporatively dried sperm after 30 d of storage at –80 °C and rehydration had

Table 2. Body weights (g; mean ± standard error of the mean) of mice produced by natural mating, ICSI with fresh spermatozoa, and ICSI with evaporatively dried spermatozoa after 30 d of storage at –80 °C (first generation)

	Natural mating	Fresh sperm	Evaporated sperm
C57BL/6 mice			
day 14	9.1 ± 0.1	9.3 ± 0.2	9.2 ± 0.2
day 28 (males)	19.5 ± 0.7	20.0 ± 0.3	20.4 ± 0.4
day 28 (females)	17.5 ± 0.5	18.1 ± 0.7	17.7 ± 0.6
day 35 (males)	23.2 ± 0.7	25.0 ± 0.4	24.6 ± 0.4
day 35 (females)	21.0 ± 0.4	20.1± 0.4	20.3 ± 0.3
day 42 (males)	26.2 ± 0.8	26.9 ± 0.4	26.6 ± 0.4
day 42 (females)	22.3 ± 0.5	21.0 ± 0.5	22.1 ± 0.4
FVB/N mice			
day 14	8.5 ± 0.2	8.6 ± 0.2	8.6 ± 0.2
day 28 (males)	21.8 ± 0.4	20.9 ± 0.5	21.8 ± 0.4
day 28 (females)	17.8 ± 0.4	18.7 ± 0.4	18.6 ± 0.3
day 35 (males)	25.0 ± 0.4	24.3 ± 0.4	24.1 ± 0.6
day 35 (females)	20.2 ± 0.4	20.9 ± 0.4	20.6 ± 0.3
day 42 (males)	27.2 ± 0.4	27.1 ± 0.4	26.9 ± 0.4
day 42 (females)	21.9 ± 0.5	22.2 ± 0.7	20.9 ± 0.4

Table 3. Litter sizes, weaning rates, and gender ratios of mice produced by natural mating, after ICSI with fresh spermatozoa, and after ICSI with evaporatively dried spermatozoa after 30 d of storage at –80 °C (second generation)

	Natural mating	Fresh sperm	Evaporated sperm
C57BL/6 mice			
No. of breeding pairs	4	4	4
No. of pups born	21	22	17
Litter size	3 to 7	3 to 7	3 to 5
Weaning rate (%)	100	100	100
Gender ratio (male/female)	1.1 (11/10)	0.8 (10/12)	0.9 (8/9)
FVB/N mice			
No. of breeding pairs	6	5	5
No. of pups born	54	44	47
Litter size	6 to 12	7 to 11	6 to 12
Weaning rate (%)	100	100	100
Gender ratio (male/female)	1.0 (27/27)	0.8 (19/25)	1.0 (24/23)

normal morphology, and even after 10 mo of storage, they were still intact and looked normal (Figure 2).

Effect of evaporative drying and frozen storage of sperm on body weight of the first generation. To determine whether evaporative drying and frozen storage of spermatozoa at –80 °C affected the growth of mice, the pups in each group were weighed on days 14, 28, 35 and 42. Because male mice grow faster than female after 2 wk of age, attention was paid to the gender of pups when measuring body weights on day 28 and after. Pups derived by ICSI using evaporatively dried spermatozoa grew normally in both the C57BL/6J and FVB/NJ strains: their body weights were not significantly different from those of the fresh sperm ICSI group and the natural mating group (Table 2).

Effect of evaporative drying and frozen storage of sperm on litter size, gender ratio, and body weight of the second generation. All of the breeding pairs of C57BL/6J and FVB/NJ mice established from offspring from the first generation of the 3 experimental groups (natural mating, fresh sperm and evaporated sperm) each had a single litter (Table 3). Statistical analysis of the mean litter sizes within each strain showed that the evaporated sperm group did not differ significantly from the fresh sperm group and the natural mating group. In addition, no significant differences in gender ratios within each strain were present between any of the groups.

The body weight of each pup in the second generation was weighed on days 14, 28, and 42 (Table 4). Statistical analysis showed that, for both strains, the mean body weights of the pups on each day in the evaporatively dried sperm group were not significantly different from those of the fresh sperm ICSI and natural mating groups, although there was a trend toward lower body weight in male C57BL/6J mice in the evaporated sperm group.

Effects of ICSI, evaporative drying, and frozen storage of sperm on the incidence of nonlethal pathologic changes and susceptibility to adventitious pathogens. The results of necropsy and histologic pathology examinations of the 2 generations of C57BL/6J and FVB/NJ mice are summarized in Table 5. The number of mice with gross lesions or noteworthy histology lesions did not vary significantly with respect to generation, group, or strain. Gross and histologic lesions were found only in C57BL/6J mice and included a 1- to 3-mm dark-red area on the tip of spleen, lymphoplasmacytic pancreatitis, mild hepatic necrosis, kidney atrophy, and mild hydronephrosis.

No evidence of infectious disease was found in any of the first-generation mice evaluated from the 3 groups from both mouse strains, and no serum antibodies to the viral pathogens examined were detected. Bacterial pathogens and parasites (pinworms, fur mites, and their ova) were absent as well.

Table 4. Body weights (g; mean ± standard error of the mean) of mice produced by natural mating, after ICSI with fresh spermatozoa, and after ICSI with evaporatively dried spermatozoa after 30 d of storage at –80 °C (second generation)

	Natural mating	Fresh sperm	Evaporated sperm
C57BL/6 mice			
day 14	8.1 ± 0.2	7.7 ± 0.1	7.5 ± 0.2
day 28 (males)	18.8 ± 0.4	18.6 ± 0.5	17.9 ± 0.4
day 28 (females)	17.4 ± 0.3	16.6 ± 0.3	16.4 ± 0.4
day 42 (males)	25.1 ± 0.7	24.7 ± 0.4	23.9 ± 0.3
day 42 (females)	19.8 ± 0.6	19.4 ± 0.3	19.1 ± 0.3
FVB/N mice			
day 14	8.1 ± 0.1	8.2 ± 0.2	8.3 ± 0.1
day 28 (males)	20.0 ± 0.4	20.9 ± 0.5	20.2 ± 0.3
day 28 (females)	17.4 ± 0.2	17.1 ± 0.5	17.6 ± 0.2
day 42 (males)	25.4 ± 0.5	24.7 ± 0.3	24.2 ± 0.3
day 42 (females)	20.5 ± 0.3	21.1 ± 0.3	20.5 ± 0.3

Table 5. Results of necropsy and histologic pathology of mice from the first (F1) and second (F2) generations

	No. of mice with lesion(s)					
	Natural mating		Fresh sperm		Evaporated sperm	
	C57BL/6	FVB/N	C57BL/6	FVB/N	C57BL/6	FVB/N
F1 gross lesion	1 of 10	0 of 10	1 of 10	0 of 10	2 of 10	0 of 10
F1 histologic lesion	2 of 10	0 of 10	0 of 10	0 of 10	1 of 10	0 of 10
F2 gross lesion	0 of 8	0 of 10	1 of 9	0 of 9	1 of 10	0 of 10
F2 histologic lesion	0 of 8	0 of 10	0 of 9	0 of 9	0 of 10	0 of 10

Discussion

Previous studies have demonstrated that trehalose on both sides of the cell plasma membrane protects mammalian cells against the potential damaging effects of evaporative drying.[4-6] Trehalose also had a protective effect on partially evaporatively dried mouse spermatozoa.[13,15] When partially evaporatively dried hybrid B6D2F1 mouse spermatozoa in trehalose–EGTA solution are stored at –80 °C, they can be kept for at least 5 mo,[13] and after rehydration, their heads successfully fertilize mouse ova by ICSI. In the present report, we demonstrated that evaporatively dried spermatozoa from inbred C57BL/6J and FVB/NJ mice can similarly be stored successfully at –80 °C.

The data presented here show that ova fertilized using ICSI with the heads of evaporatively dried spermatozoa from inbred C57BL/6J and FVB/NJ mice, stored for 1 mo at –80 °C, can develop into healthy and reproductively sound adult mice. No significant differences were found in embryo transfer rate (number of pups born/number of embryos transferred), litter size, weaning rate, body weight, number of pathologic lesions, and amount of contamination with pathogens among the 3 experimental groups of natural mating, ICSI using fresh spermatozoa, and ICSI using evaporatively dried spermatozoa. The present study also has failed to demonstrate any significant effect of using evaporatively dried mouse spermatozoa, stored at a low temperature, to fertilize ova and on the growth and development of offspring over 2 generations of breeding.

ICSI is a remarkably effective assisted reproductive technique which bypasses all natural sperm selection processes (cumulus penetration, zona binding, acrosome reaction, zona penetration, oolemma fusion, and so forth) by injection of a single spermatozoon directly into the ooplasm.[14] In mice, the head of the spermatozoon is separated from its tail by piezoelectric pulses applied to the neck region. Only the head of the spermatozoon, but not its long tail, is injected into the ovum, to reduce the amount of introduced medium. The results of necropsy and histologic pathology of mice produced by ICSI using fresh and evaporatively dried and stored spermatozoa demonstrate that the procedures had no deleterious effects on the offspring spanning 2 generations of C57BL/6J and FVB/NJ mice. The results further show that evaporative drying and storage of spermatozoa at –80 °C had no effect on 2 generations of offspring after spermatozoa were hydrated and used to fertilize mouse ova by using ICSI.

This study is the first to demonstrate that the use of ICSI and dried spermatozoa, stored at a low temperature, does not result in any noteworthy biologic, reproductive, or pathologic abnormalities in offspring. These results are especially relevant to sperm preservation in the C57BL/6J strain of mouse. The C57BL/6J strain is the most widely used strain for studies on the mouse and human genomes, and many genetic mutants are maintained on this background.[28] Unfortunately the spermatozoa of the C57BL/6J strain are very susceptible to freezing,[8,18,19,23] and this drawback has complicated archiving and preserving mutant mouse lines on the C57BL/6J background. Our results show that the combination of evaporative drying and frozen storage at –80 °C is a viable approach for preserving spermatozoa of this mouse strain. Further research is needed to determine whether evaporative drying of mouse spermatozoa of wild-type and mutant C57BL/6J and other inbred strains can be preserved for longer times (years) without loosing the ability to fertilize ova and produce normal offspring.

Acknowledgments

The project described was supported by grants R24 RR018934 and U42 RR14905 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). Its contents are solely the responsibility of the authors and do not necessarily represent

the official views of NCRR or NIH. This project was also supported by the UC Davis Mouse Biology Program.

References

1. **Bath ML.** 2003. Simple and efficient in vitro fertilization with cryopreserved C57BL/6J mouse sperm. Biol Reprod **68:**19–23.
2. **Bhowmick S, Zhu L, McGinnis L, Lawitts J, Nath BD, Mehmet T, Biggers JD.** 2003. Desiccation tolerance of spermatozoa dried at ambient temperature: production of fetal mice. Biol Reprod **68:**1779–1786.
3. **Biggers JD, McGinnis, LK, Raffin M.** 2000. Amino acids and preimplantation development of the mouse in protein-free potassium simplex optimized medium. Biol Reprod **63:**281–293.
4. **Buchanan SS, Menze MA, Hand SC, Pyatt DW, Carpenter JF.** 2005. Cryopreservation of human hematopoietic stem and progenitor cells loaded with thehalose: transient permeabilization via the adenosine triphosphate-dependent P2Z receptor channel. Cell Preservation Technology **3:**212–222.
5. **Chen T, Acker JP, Eroglu A, Cheley S, Bayley H, Fowler A, Toner M.** 2001. Beneficial effect of intracellular trehalose on the membrane integrity of dried mammalian cells. Cryobiology **43:**168–181.
6. **Eroglu A, Russo MJ, Bieganski R, Fowler A, Cheley S, Bayley H, Toner M.** 2000. Intracellular trehalose improves the survival of cryopreserved mammalian cells. Nature Biotechnol **18:**163–167.
7. **Kaneko T, Nakagata N.** 2005. Relation between storage temperature and fertilizing ability of freeze-dried mouse spermatozoa. Comp Med **55:**140–144.
8. **Kawai Y, Hata T, Suzuki O, Matsuda J.** 2006. The relationship between sperm morphology and in vitro fertilization in mice. J Reprod Dev **52:**561–568.
9. **Kawase Y, Araya H, Kamada N, Jishage K, Suzuki H.** 2005. Possibility of long-term preservation of freeze-dried mouse spermatozoa. Biol Reprod **72:**568–573.
10. **Kimura Y, Yanagimachi R.** 1995. Intracytoplasmic sperm injection in the mouse. Biol Reprod **52:**709–720.
11. **Kusakable H, Szczygiel MA, Whittingham DG, Yanagimachi R.** 2001. Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa. Proc Nat Acad Sci U S A **98:**13501–13506.
12. **Lawitts JA, Biggers JD.** 1993. Culture of preimplantation embryos. Methods Enzymol **225:**153–164
13. **Li MW, Biggers JD, Elmoazzen HY, Toner M, McGinnis L, Lloyd KCK.** 2007. Long term storage of mouse spermatozoa after evaporative drying. Reproduction **133:**919–929.
14. **Li MW, Lloyd KCK.** 2006. Intracytoplasmic sperm injection (ICSI) in the mouse. In: Pease S, Lois C, editors. Principles and practice: mammalian and avian transgenesis—new approaches. Springer: Verlag Berlin Heidelberg. p 23–40.
15. **McGinnis L, Zhu L, Lawitts J, Bhowmick S, Mehmet T, Biggers JD.** 2005. Mouse sperm desiccated and stored in trehalose medium without freezing. Biol Reprod **73:**627–633.
16. **Nagy A, Gertsenstein M, Vintersten K, Behringer R.** 2003. Vasectomy for generation of sterile males. In: Nagy A, Gertsenstein M, Vintersten K, Behringer R, editors. Manipulating the mouse embryo: a laboratory manual, 3rd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press. p 258–260.
17. **Nakagata N.** 2000. Cryopreservation of mouse spermatozoa. Mamm Genome **11:**572–576.
18. **Nakagata N, Okamoto M, Ueda O, Suzuki H.** 1997. Positive effect of partial zona-pellucida dissection on the in vitro fertilizing capacity of cryopreserved C57BL/6J transgenic mouse spermatozoa of low motility. Biol Reprod **57:**1050–1055.
19. **Nishizono H, Shioda M, Takeo T, Irie T, Nakagata N.** 2004. Decrease of fertilizing ability of mouse spermatozoa after freezing and thawing is related to cellular injury. Biol Reprod **71:**973–978.
20. **Okuyama M, Isogai S, Saga M, Hamada H, Ogawa S.** 1990. In vitro fertilization (IVF) and artificial insemination (AI) by cryopreserved spermatozoa in mouse. J Fert Implant (Tokyo) **7:**116–119.
21. **Songsasen N, Leibo SP.** 1997. Cryopreservation of mouse spermatozoa. II. Relationship between survival after cryopreservation and osmotic tolerance of spermatozoa from three strains of mice. Cryobiology **35:**255–269.
22. **Szczygiel MA, Kusakabe H, Yanagimachi R, Whittingham DG.** 2002. Intracytoplasmic sperm injection is more efficient than in vitro fertilization for generating mouse embryos from cryopreserved spermatozoa. Biol Reprod **67:**1278–1284.
23. **Sztein JM, Farley JS, Mobraaten LE.** 2000. In vitro fertilization with cryopreserved inbred mouse sperm. Biol Reprod **63:**1774–1780.
24. **Tada N, Sato M, Yamanoi J, Mizorogi T, Kasai K, Ogawa S.** 1990. Cryopreservation of mouse spermatozoa in the presence of raffinose and glycerol. J Reprod Fert **89:**511–516.
25. **Thomton CG, Brown SD, Glenister PH.** 1999. Large numbers of mice established by in vitro fertilization with cryopreserved spermatozoa: implication and applications for genetic resource banks, mutagenesis screens, and mouse backcrosses. Mamm Genome **10:**987–992.
26. **Wakayama T, Yanagimachi R.** 1998. Development of normal mice from oocytes injected with freeze-dried spermatozoa. Nature Biotech **16:**639–641.
27. **Ward MA, Kaneko T, Kusakabe H, Biggers JD, Whittingham DG, Yanagimachi R.** 2003. Long-term preservation of mouse spermatozoa after freeze-drying and freezing without cryoprotection. Biol Reprod **69:**2100–2108.
28. **Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P, Antonarakis SE, Attwood J, Baertsch R, Bailey J, Barlow K, Beck S, Berry E, Birren B, Bloom T, Bork P, Botcherby M, Bray N, Brent MR, Brown DG, Brown SD, Bult C, Burton J, Butler J, Campbell RD, Carninci P, Cawley S, Chiaromonte F, Chinwalla AT, Church DM, Clamp M, Clee C, Collins FS, Cook LL, Copley RR, Coulson A, Couronne O, Cuff J, Curwen V, Cutts T, Daly M, David R, Davies J, Delehaunty KD, Deri J, Dermitzakis ET, Dewey C, Dickens NJ, Diekhans M, Dodge S, Dubchak I, Dunn DM, Eddy SR, Elnitski L, Emes RD, Eswara P, Eyras E, Felsenfeld A, Fewell GA, Flicek P, Foley K, Frankel WN, Fulton LA, Fulton RS, Furey TS, Gage D, Gibbs RA, Glusman G, Gnerre S, Goldman N, Goodstadt L, Grafham D, Graves TA, Green ED, Gregory S, Guigo R, Guyer M, Hardison RC, Haussler D, Hayashizaki Y, Hillier LW, Hinrichs A, Hlavina W, Holzer T, Hsu F, Hua A, Hubbard T, Hunt A, Jackson I, Jaffe DB, Johnson LS, Jones M, Jones TA, Joy A, Kamal M, Karlsson EK, Karolchik D, Kasprzyk A, Kawai J, Keibler E, Kells C, Kent WJ, Kirby A, Kolbe DL, Korf I, Kucherlapati RS, Kulbokas EJ, Kulp D, Landers T, Leger JP, Leonard S, Letunic I, Levine R, Li J, Li M, Lloyd C, Lucas S, Ma B, Maglott DR, Mardis ER, Matthews L, Mauceli E, Mayer JH, McCarthy M, McCombie WR, McLaren S, McLay K, McPherson JD, Meldrim J, Meredith B, Mesirov JP, Miller W, Miner TL, Mongin E, Montgomery KT, Morgan M, Mott R, Mullikin JC, Muzny DM, Nash WE, Nelson JO, Nhan MN, Nicol R, Ning Z, Nusbaum C, O'Connor MJ, Okazaki Y, Oliver K, Overton-Larty E, Pachter L, Parra G, Pepin KH, Peterson J, Pevzner P, Plumb R, Pohl CS, Poliakov A, Ponce TC, Ponting CP, Potter S, Quail M, Reymond A, Roe BA, Roskin KM, Rubin EM, Rust AG, Santos R, Sapojnikov V, Schultz B, Schultz J, Schwartz MS, Schwartz S, Scott C, Seaman S, Searle S, Sharpe T, Sheridan A, Shownkeen R, Sims S, Singer JB, Slater G, Smit A, Smith DR, Spencer B, Stabenau A, Stange-Thomann N, Sugnet C, Suyama M, Tesler G, Thompson J, Torrents D, Trevaskis E, Tromp J, Ucla C, Ureta-Vidal A, Vinson JP, Von Niederhausern AC, Wade CM, Wall M, Weber RJ, Weiss RB, Wendl MC, West AP, Wetterstrand K, Wheeler R, Whelan S, Wierzbowski J, Willey D, Williams S, Wilson RK, Winter E, Worley KC, Wyman D, Yang S, Yang SP, Zdobnov EM, Zody MC, Lander ES.** 2002. Mouse genome sequencing consortium: initial sequencing and comparative analysis of the mouse genome. Nature **420:**520–562.
29. **Yokoyama M, Akiba H, Katsuki M, Nomura T.** 1990. Production of normal young following transfer of mouse embryos obtained by in vitro fertilization using cryopreserved spermatozoa. Exp Anim **39:**126–128.