

Long-Term Preservation of Mouse Spermatozoa after Freeze-Drying and Freezing Without Cryoprotection¹

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ABSTRACT

The widespread production of mice with transgenes, disrupted genes and mutant genes, has strained the resources available for maintaining these mouse lines as live populations, and dependable methods for gamete and embryo preservation in these lines are needed. Here we report the results of intracytoplasmic sperm injection (ICSI) with spermatozoa freeze-dried or frozen without a cryoprotectant after storage for periods up to 1.5 years. Freeze-dried samples were stored at 4°C. Samples frozen without cryoprotection were maintained at -196°C. After storage, spermatozoa were injected into the oocytes by ICSI. Zygotic chromosomes and fetal development at Day 15 of gestation were examined after 0, 1, 3, 6, 9, and 12 mo of sperm storage. When fresh spermatozoa were used for ICSI, 96% of resultant zygotes contained normal chromosomes, and 58% of two-cell embryos transferred developed to normal viable fetuses. Similar results were obtained when spermatozoa were frozen without cryoprotection and then used for ICSI (87% and 45%, respectively; $P > 0.05$) and after 12 mo of sperm storage (mean of six endpoints examined: 87% and 52%, respectively; $P > 0.05$). Freeze-drying decreased the proportion of zygotes with normal karyotypes (75% vs. 96%; $P < 0.001$) and the proportion of embryos that developed into fetuses (35% vs. 58%; $P < 0.001$), but similar to freezing, there was no further deterioration during 12 mo of storage (mean of six endpoints examined: 68% and 34%, respectively; $P > 0.05$). Live offspring were obtained from both freeze-dried and frozen spermatozoa after storage for 1.5 yr. The results indicate that 1) the freeze-drying procedure itself causes some abnormalities in spermatozoa but freezing without cryoprotection does not and 2) long-term storage of both frozen and freeze-dried spermatozoa is not deleterious to their genetic integrity. Freezing without cryoprotection is highly successful, simple, and efficient but, like all routine sperm storage methods, requires liquid nitrogen. Liquid nitrogen is also required for freeze-drying, but sperm can then be stored

at 4°C and shipped at ambient temperatures. Both preservation methods are successful, but rapid freezing without cryoprotection is the preferred method for preservation of spermatozoa from mouse strains carrying unique genes and mutations.

assisted reproductive technology, embryo, gamete biology, in vitro fertilization, sperm

INTRODUCTION

Sperm cryopreservation has contributed greatly to animal breeding and assisted conception in humans since Polge et al. [1] first reported in 1949 that glycerol protected fowl spermatozoa from freezing injury. Today, much of the basic research in mammalian genetics and early development is undertaken with the mouse. The production of mice with transgenes, disrupted genes, or mutant genes is commonplace, resulting in an abundance of valuable genomes that need to be conserved for future use. The numerous mutant lines have put a strain on both the financial and physical resources available to maintain them as live populations. Efficient and dependable methods for gamete and embryo cryopreservation are needed to avoid the inadvertent loss of this unique material through disease or other hazard. Preservation of gametes/embryos can provide an effective means of distributing these novel genetic models throughout the biomedical research community. Spermatozoa are produced in much larger numbers compared with oocytes and embryos and therefore the conservation of genes within the haploid sperm genome is an attractive alternative to oocyte and embryo storage for the preservation of specific mutations or inserted genes. Sperm cryopreservation is more cost effective and less labor intensive than embryo freezing. Furthermore, with the advent of intracytoplasmic sperm injection (ICSI) many breeding problems exhibited by defective male reproductive function in mutant and transgenic lines, e.g., low sperm motility or concentration and/or abnormal sperm morphology, can be overcome [2–5].

Successful cryopreservation of mouse spermatozoa was achieved relatively recently [6–9]. Many techniques have been described in the literature during the last 13 years, claiming varying degrees of success [7, 10–12]. Nakagata and coworkers have published several reports [13–17] on the successful cryopreservation of spermatozoa from a variety of strains and transgenic stocks using raffinose and skim milk for cryoprotection. Although some laboratories have adopted what is now generally known as Nakagata's freezing method [18–20], the technique is not universally successful for all strains of mice. Mouse spermatozoa are highly sensitive to several types of mechanical stress, e.g., free oxygen radicals, osmotic changes, and regimens for cooling and warming [21–25]. Although the spermatozoa

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from some strains appear undamaged by exposure to cryoprotectant(s) and freezing procedure(s), those of other strains (mostly inbred) are seriously damaged as indicated by very low rates of fertilization [7, 16, 20].

Difficulties with conventional methods of sperm cryopreservation in the mouse have led to exploration of alternative methods such as freeze-drying [26], freezing without cryoprotection [27], and more recently desiccation [28]. The nonconventional methods of sperm preservation differ from conventional approaches in that the spermatozoa are not alive (motile with intact plasma membranes) after storage. Thus, the micromanipulation technique of ICSI is required to achieve fertilization.

The uniqueness of these nonconventional approaches is that they bypass the major difficulty encountered by the conventional methods, i.e., cryodamage. Cryodamaged spermatozoa are unable to fertilize oocytes both *in vivo* and *in vitro*. Freeze-drying and freezing without cryoprotection destroy the structural integrity of spermatozoa. Sperm plasma membranes are extensively damaged, and therefore spermatozoa are dead in the conventional sense. Although general structures of spermatozoa are damaged by these nonconventional means of sperm preservation, sperm DNA [26, 27, 29] and intracellular factors that activate the oocyte [30, 31] can be preserved.

Recently, we reported that a simple Tris-HCl-buffered solution with high concentration of the calcium chelating agent EGTA, normally used for the preparation of DNA from eukaryotic cells, better maintains chromosome integrity of spermatozoa during freeze-drying than does ordinary cell culture medium [29, 32]. In the present study we compared fertilizing capacity and chromosome integrity of mouse spermatozoa that were either freeze-dried or frozen without a cryoprotectant and then stored for up to 18 mo. We examined 1) genetic integrity of spermatozoa by the analysis of chromosome complement of ICSI-injected oocytes prior to the first cleavage division and 2) the developmental potential of embryos generated from the preserved spermatozoa by assessing fetal development after embryo transfer.

MATERIALS AND METHODS

Chemicals

Mineral oil was purchased from Squibb and Sons (Princeton, NJ), and eCG and hCG were from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Animals

Mice were obtained at 6 wk of age from the following sources: B6D2F₁ (C57BL/6J × DBA/2) and CD-1 from the National Cancer Institute (Raleigh, NC) and FVB from Jackson Laboratory (Bar Harbor, ME). Epididymal spermatozoa were collected from 8- to 12-wk-old FVB males. Mature oocytes were obtained from 8- to 12-wk-old B6D2F₁ hybrid females. Recipients of two-cell embryos were 8- to 16-wk-old random-bred CD-1 females. The mice were fed *ad libitum* with a standard diet and maintained in a temperature and light-controlled room (22°C, 14L:10D; light starting at 0700 h), in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council (DHEW publication 80-23, revised in 1985). The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii.

Solution for Sperm Preservation

The EGTA Tris-HCl-buffered solution for suspending the spermatozoa for freeze-drying and rapid freezing without cryoprotection consisted of

50 mM EGTA, 50 mM NaCl, and 10 mM Tris-HCl buffer [29]. The pH was adjusted to 8.2–8.5 by adding a small quantity of 1 M NaOH. This solution was stored at 4°C for no more than 1 wk before use.

Media

Oocyte collection and subsequent oocyte manipulation, including microinjection, were carried out in HEPES-buffered CZB medium (HEPES-CZB [33]), which was maintained in air. Sperm-injected oocytes and embryos were cultured in CZB medium [34] supplemented with 5.56 mM glucose, which was maintained in an atmosphere of 5% CO₂ in air.

Sperm Collection and Preservation

One milliliter of EGTA Tris-HCl-buffered solution was placed in a 1.5-ml polypropylene microcentrifuge tube (Fisher Scientific, Pittsburgh, PA) and warmed to 37°C. The two epididymides were removed from an FVB male, and dense sperm masses were expressed from the caudal region of each epididymis after puncturing the epididymis with a pair of sharp forceps. The sperm masses from both epididymides were gently placed beneath the EGTA Tris-HCl-buffered solution in a microcentrifuge tube and kept at 37°C for 10 min to allow spermatozoa to disperse into the solution. The upper 800 µl of the sperm suspension was carefully collected and divided, and part of the solution (700 µl) was used for freeze-drying and the other part (100 µl) was used for rapid freezing without cryoprotection.

Freeze-drying of spermatozoa was carried out as described previously [29]. Seven hundred microliters of sperm suspension was divided into seven 100-µl aliquots that were then transferred into seven longneck glass ampules (no. 651506; Wheaton, Millville, NJ). The ampules were plunged into liquid nitrogen for 20 sec and then connected to the freeze-drying machine (Freeze-Dry Systems; Labconco, Kansas City, MO). Four hours later, the ampules were flame-sealed. The pressure inside the ampules was 30–33 × 10⁻³ mbar at the time of sealing. Ampules were stored in a refrigerator (4°C) until use. Immediately before ICSI, an ampoule with freeze-dried spermatozoa was brought to room temperature and opened, and spermatozoa were hydrated by adding 100 µl of sterile distilled water.

For rapid freezing without cryoprotection, 10-µl samples of spermatozoa dispersed in EGTA Tris-HCl-buffered solution were loaded into 0.25-ml straws (Edwards Innovations, Spring Valley, VA). Each straw was sealed with Critoseal (Oxford Labware, St. Louis, MO) and placed in a plastic holder. In preliminary experiments, the straws were transferred directly from room temperature to liquid nitrogen (LN₂) at -196°C, resulting in the total disintegration of many of the straws because of rapid pressure changes occurring during this procedure. The problem was solved by placing the straws in a plastic holder on the surface of the LN₂ for 10 min before immersion. Immediately before ICSI, a straw was removed from the storage container and thawed at room temperature (~25°C) for 5 min before expressing the contents into a Petri dish.

Oocyte Collection

Mice 8–12 wk old were induced to superovulate by consecutive injections of 5 IU eCG and 5 IU hCG given 48 h apart. Oviducts were removed 14–15 h after the injection of hCG and placed in HEPES-CZB medium in a Petri dish. The cumulus-oocyte complexes were released from the oviducts into 0.1% bovine testicular hyaluronidase (300 USP units/mg) in HEPES-CZB medium to disperse cumulus cells. Cumulus-free oocytes were washed with HEPES-CZB medium and used immediately for ICSI. The oocytes were divided into two groups, one for ICSI with freeze-dried spermatozoa and the other for ICSI with spermatozoa rapidly frozen without cryoprotection.

Intracytoplasmic Sperm Injection

The ICSI procedure used was that of Kimura and Yanagimachi [33], with some modifications. A small drop of sperm suspension was mixed thoroughly with an equal volume of HEPES-CZB containing 12% (w/v) polyvinylpyrrolidone (360 kDa) immediately before ICSI, which was performed using either a micromanipulator (Micromanipulator TransferMan; Eppendorf, Hamburg, Germany) or a manipulator (Narishige, Tokyo, Japan) with a piezo-electric actuator (PMM Controller, model PMAS-CT150; Prima Tech, Tsukuba, Japan). A single spermatozoon was drawn, tail first, into the injection pipette and moved back and forth until the head-midpiece junction (neck) was at the opening of the injection pipette. The head was separated from the midpiece by applying one or more piezo pulses. After discarding the tail, the head was redrawn into the pipette and injected immediately into an oocyte. Injections were performed in HEPES-

CZB within 1 h after oocyte collection and sperm reconstitution. Sperm-injected oocytes were transferred into CZB medium and examined about 6 h after ICSI to assess survival and activation. An oocyte was recorded as activated when it contained two well-developed pronuclei and the distinct second polar body. Activated oocytes were divided into two groups, one for chromosome analysis and the other for further culture and embryo transfer.

Embryo Culture and Transfer

Embryos reaching the two-cell stage were transferred to the oviducts (5–10 embryos/oviduct) of CD-1 females mated during the previous night with vasectomized CD-1 males. The number of implantation sites and fetuses were recorded on Day 15 of gestation. This day was chosen to provide information on the extent of early embryonic loss after implantation. Normal Day 15 fetuses rarely fail to develop to full term.

Pseudopregnant females receiving two-cell embryos generated from freeze-dried and frozen spermatozoa after 1.5 years of storage delivered and reared the progeny, which were subsequently bred to examine normality and fertility.

Chromosomal Analysis

Examination of chromosomes in one-cell embryos after ICSI has been used previously to assess the normality of the paternal complement [29, 35–37]. Fertilized oocytes were transferred after 6–8 h of culture into CZB containing 0.006 $\mu\text{g/ml}$ vinblastine, which was added to inhibit syngamy. Between 19 and 21 h after ICSI, oocytes were treated with 1% pronase (1000 tyrosine units/mg; Kaken Pharmaceuticals, Tokyo, Japan) for 5 min at room temperature to soften zonae pellucidae. Then the oocytes were treated with hypotonic solution (1:1 mixture of 1% sodium citrate:30% fetal bovine serum) for 5 min at 37°C or 10 min at 25°C. Chromosomes were spread on clean glass slides by the gradual fixation/air-drying method [38]. The preparations were stained with 2% Giemsa (Merck, Darmstadt, Germany) in PBS (pH 6.8) for 10 min for conventional chromosome analysis. The chromosomes of a spermatozoon were considered normal when an egg contained 40 normal metaphase chromosomes. It was not always possible to distinguish between chromosomes of paternal and maternal origin. However, because oocyte chromosomes seldom show structural aberrations at first cleavage metaphase after parthenogenetic activation (unpublished observations) [29, 35–37], any abnormal chromosomes within fertilized oocytes were considered to be of sperm origin.

Shipment

To assess whether spermatozoa frozen without a cryoprotectant and stored in LN_2 can be shipped on dry ice, we evaluated paternal chromosomes at the four stages of the shipment process. Spermatozoa were obtained from B6D2F₁ males and preserved by rapid freezing without cryoprotection as described above. A portion of the sperm suspension was used for ICSI prior to freezing, as fresh sperm control, and three straws containing sperm suspension were frozen in LN_2 . After at least 24 h of storage, one straw was thawed and spermatozoa were used for ICSI, and two straws were quickly transferred into a Styrofoam container with dry ice. The container was taped and wrapped as for shipment and kept on the bench at 25°C for 48 h. One straw was then thawed, and spermatozoa were used for ICSI. The remaining last straw was transferred back to LN_2 and stored for at least 24 h before being thawed.

Experimental Design

Experiments were designed to assess fertilization, implantation, and fetal development after the injection of oocytes with freeze-dried spermatozoa and spermatozoa rapidly frozen without a cryoprotectant. The frequency of normal karyotypes was examined before the first cleavage division. Samples of preserved spermatozoa were examined after storage for 0 (1–7 days), 1, 3, 6, 9, and 12 mo. Epididymal spermatozoa were obtained from five males, and samples from each male were separately freeze-dried and rapidly frozen. Fresh sperm samples from all males were used as controls. The oocytes for ICSI were obtained from several females, pooled, and divided for ICSI with freeze-dried spermatozoa or with spermatozoa frozen without cryoprotection. Injections with freeze-dried and frozen spermatozoa were done side by side by two persons. In each experiment, the oocytes that survived ICSI and became activated were divided into two groups, one for chromosome analysis and the other for culture to the two-cell stage before embryo transfer to surrogate mothers.

Statistical Analysis

An ANOVA is not valid for the analysis of the data presented here because the data are unbalanced, with group sizes varying from cell to cell, and the data are sparse, with very few observations within some cells. Thus, all the data were analyzed by logistic regression analysis, in which the proportions are assumed to follow a binomial distribution. This type of analysis has been used elsewhere and is particularly useful when the data are sparse and unbalanced [39]. In this analysis, the observed proportions (p) are transformed into logits defined by: $y = \text{logit } p = \ln[p/(1 - p)]$. The variables in the factorial experiment are the six periods of storage (0, 1, 3, 6, 9, 12 mo) and two preservation techniques (freeze-drying and rapid freezing without cryoprotection). In the statistical analyses, freeze-drying is coded 0 and freezing without cryoprotection is coded 1. The responses of the factorial experiment for each mouse have been represented by the following model: $y = b_0 + b_1x_1 + b_2x_2$ (model 1), where x_1 is the time of storage, x_2 is the method of preservation, b_0 is the intercept, b_1 is the linear regression on storage time, and b_2 is the regression on preservation method. This model assumes that there is no interaction between time of storage and method of preservation. Thus, the regression lines for each method are assumed to be parallel. When $x_2 = 0$, model 1 reduces to $y = b_0 + b_1x_1$. Thus, b_2 is the vertical distance between the two regression lines. The Hosmer-Lemeshaw test was used to examine the goodness of fit of model 1 for all data analyzed.

The methods for performing logistical regression analysis are available in the computer program LogXact 5 (Cytel Software Corporation, Cambridge, MA). Three methods for computing the regression coefficients are available: asymptotic, exact, and Monte Carlo. The asymptotic method is adequate when the observed proportions lie between 0.05 and 0.95 and when the group sizes are sufficient (>5). When these conditions are not satisfied, then the exact method should be used. This method was applied in all the calculations for analyzing data in this study.

RESULTS

The data were obtained in a 2×6 factorial experiment that was replicated using sperm from five mice. Control data were obtained with fresh spermatozoa from the five males. Several types of observations were made: number of injected oocytes, number of oocytes that survived ICSI, number of cultured oocytes, number of two-cell embryos, number of oocytes with normal karyoplasts, number of embryos transferred, number of embryos that implanted, and number of embryos that developed to fetuses (Table 1 and Figs. 1 and 2). These data are binary and categorical. Certain ratios between these categories were selected: the number of normal karyotypes from number of karyotypes analyzed (genetic integrity of spermatozoa), the number of two-cell embryos from the number of oocytes cultured (fertilization rate), the number of implantation sites from the number of two-cell embryos transferred (implantation rate), and the number of fetuses from the number of two-cell embryos transferred (fetal rate).

Genetic Integrity of Spermatozoa

The genetic (chromosome) integrity of spermatozoa was assessed based on the proportion of normal karyoplasts in one-cell embryos produced by ICSI with freeze-dried and frozen spermatozoa. The Hosmer-Lemeshaw test was used to examine the goodness of fit of model 1, and the estimates of the regression coefficients and their standard errors were made for each of the five males (Table 2). The probability of departure from the model is <0.05 for all mice, and therefore model 1 fits all sets of data. The regressions on time for all mice were not significantly different from zero ($P \geq 0.05$). The regressions are homogenous ($\chi^2 = 2.373$, $df = 4$, $P = 0.632$), and the weighted mean ($\pm\text{SEM}$) is 0.0164 ± 0.0229 . Thus, the incidence of normal karyoplasts is the same for all storage times (Fig. 1). The regressions on method of preservation are all significant with the exception of mouse 2. In this case, the probability falls

TABLE 1. Long-term preservation of mouse spermatozoa after freeze-drying and freezing without cryoprotection. The data from the five males have been combined for each time of storage.

Treatment	Storage time (mo)	ICSI		Chromosome analysis			Embryonic development				
		No. oocytes injected	No. (%) oocytes survived	No. oocytes for chromosome analysis	No. karyo-plates examined	No. (%) normal karyotypes	No. cultured oocytes	No. (%) two-cell embryos	No. two-cell embryos transferred	Day 15 gestation ^a	
										No. (%) implants ^b	No. (%) fetuses
None		281	251 (89)	117	94	90 (96)	134	118 (88)	118	92 (78)	69 (58)
Freeze-drying	0	170	155 (91)	75	52	39 (75)	80	73 (91)	63	47 (75)	22 (35)
	1	207	181 (87)	96	79	51 (65)	85	74 (87)	26	12 (46)	5 (19)
	3	168	147 (88)	73	48	33 (69)	74	65 (88)	53	32 (60)	16 (30)
	6	191	161 (84)	77	69	49 (71)	84	71 (85)	54	34 (63)	20 (37)
	9	166	140 (84)	69	55	42 (76)	71	58 (82)	49	41 (84)	19 (39)
	12	168	136 (81)	69	62	35 (56)	67	60 (90)	60	38 (63)	22 (37)
	Total	1070	920 (86)	459	365	249 (68)	461	401 (87)	305	204 (67)	104 (34)
Freezing without cryoprotection	0	175	158 (90)	76	58	51 (88)	82	71 (87)	56	34 (61)	25 (45)
	1	167	151 (90)	72	54	48 (89)	79	68 (86)	54	25 (46)	17 (31)
	3	146	123 (84)	60	53	45 (85)	63	52 (83)	52	47 (90)	35 (67)
	6	162	135 (83)	67	53	51 (96)	68	62 (91)	48	42 (88)	26 (54)
	9	153	126 (82)	56	49	45 (92)	70	58 (83)	58	47 (81)	30 (52)
	12	137	107 (78)	55	48	41 (85)	52	49 (94)	43	35 (81)	30 (70)
	Total	940	800 (85)	386	315	281 (89)	414	360 (87)	311	231 (74)	163 (52)

^a Percentage calculated from two-cell embryos transferred.

^b Implants include both live fetuses and resorption sites.

just short of significance. Nevertheless, the regressions are homogenous ($\chi^2 = 4.124$, $df = 4$, $P = 0.389$) and the weighted mean is 1.372 ± 0.223 . Thus, when the data are combined for all mice, the proportion of normal karyotypes in oocytes fertilized with spermatozoa frozen without cryoprotection (89%) is significantly higher than that in ova fertilized with freeze-dried spermatozoa (68%) but similar to the proportion of normal karyotypes found in oocytes after ICSI with fresh spermatozoa (96%).

Fertilization

The proportion of oocytes cultured that reached the two-cell stage reflects the rate of fertilization. The Hosmer-Lemeshaw test showed that model 1 ($y = b_0 + b_1x_1 + b_2x_2$) is adequate to fit the data for each mouse. The five regressions on time of storage are not significantly different from zero ($P \geq 0.05$). The five regressions on preservation methods are homogenous ($\chi^2 = 5.335$, $df = 4$, $P = 0.255$), and

the weighted mean (\pm SEM) is -0.0007 ± 0.0248 . Thus, with both methods of preservation, there is no significant effect of time on the proportion of two-cell embryos that developed from the cultured oocytes (Table 1). None of the regressions on method of storage are significant ($P \geq 0.05$). The regressions are homogeneous ($\chi^2 = 4.866$, $df = 4$, $P = 0.301$), and the weighted mean is -0.010 ± 0.207 . When the data were combined for all males, there was no significant difference between the proportion of two-cell embryos developing from oocytes produced with freeze-dried spermatozoa, spermatozoa frozen without cryoprotection, and fresh spermatozoa (87%, 87%, and 88%, respectively).

Embryo Implantation

When the rate of implantation of embryos produced from freeze-dried and frozen spermatozoa was examined with the Hosmer-Lemeshaw test, the model fit the data from

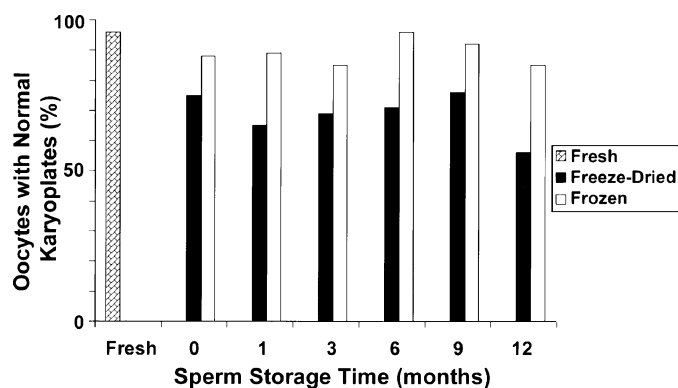


FIG. 1. Chromosome integrity of freeze-dried and rapidly frozen mouse spermatozoa assessed by the proportion of ICSI oocytes with normal chromosomes. At each time point, data from five different males were pooled. Spermatozoa used for ICSI before preservation (fresh sperm) served as controls.

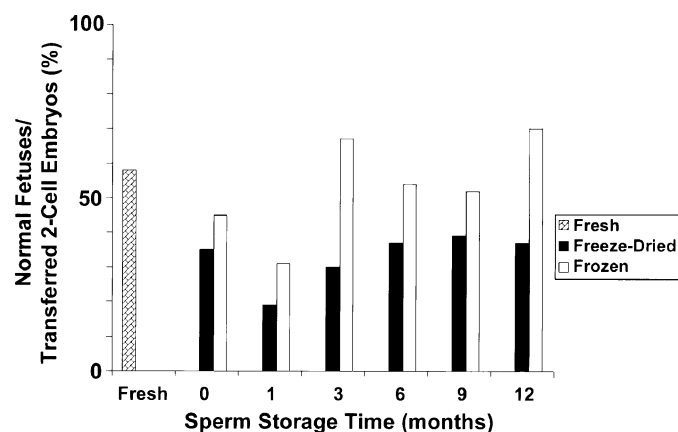


FIG. 2. Postimplantation development of mouse embryos obtained by ICSI with freeze-dried and rapidly frozen spermatozoa. At each time point, data from five different males were pooled. Spermatozoa used for ICSI before preservation (fresh sperm) served as controls.

TABLE 2. Goodness-of-fit test and the estimates of the regression coefficients and their SEMs made for each of the five males examined using the Hosmer-Lemeshaw test for the analysis of genetic integrity of mouse spermatozoa after freeze-drying and freezing without cryoprotection.

Mouse	Hosmer-Lemeshaw test (<i>P</i>)	Regression on time of storage			Regression on preservation method		
		<i>b</i> ₁ ^a	SEM	<i>P</i> ^b	<i>b</i> ₂ ^c	SEM	<i>P</i> ^b
1	0.800	-0.0091	0.0476	0.864	2.1325	0.5166	4.08E ⁻⁶
2	0.657	-0.0830	0.0517	0.111	0.8503	0.4353	0.076
3	0.465	0.0188	0.0489	0.725	1.5720	0.4860	0.0008
4	0.990	0.0229	0.0655	0.759	1.4935	0.6650	0.021
5	0.318	-0.0215	0.0473	0.663	1.1089	0.4664	0.0213

^a Linear regression on storage time.

^b Probability that the regression coefficients are significantly different from zero.

^c Regression on preservation method.

only one of the five males. The lack of fit could be due to the assumption that the two regressions on time of storage corresponding to the two methods of preservation are parallel. To test this assumption, the following model was fit to include an interaction term: $y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2$ (model 2). This model also failed the Hosmer-Lemeshaw test. The overall conclusion is that there is extra random variability in the data due to other causes. To be able to analyze the results properly, the data were plotted (Fig. 3). There was large variation among males. However, inspection of the data from each of the five mice gives no indication that the effect of storage time and method of preservation affected the proportion of transferred embryos that implanted. Overall, the proportion of embryos implanting was significantly lower for two-cell embryos derived from freeze-dried spermatozoa than for embryos derived

from fresh spermatozoa (67% vs. 78%; $P < 0.001$) or from spermatozoa frozen without cryoprotection (67% vs. 74%; $P < 0.001$). However, implantation of embryos derived from fresh spermatozoa was also significantly higher than that for embryos derived from spermatozoa frozen without cryoprotection (74% vs. 78%; $P < 0.01$).

Fetal Development

The Hosmer-Lemeshaw test showed that model 1 was adequate to fit the data for all five males (Table 3). Two of the five estimates of the regression on time of storage are significantly positive (males 2 and 5), but the other regressions are not. Further tests showed that the five estimates are not homogenous ($\chi^2 = 13.603$, $df = 4$, $P = 0.0087$). Thus the effects of time of storage are not the same for all

FIG. 3. Effect of storage time and method of preservation on the proportion of transferred mouse embryos that implanted, evaluated independently for the five males.

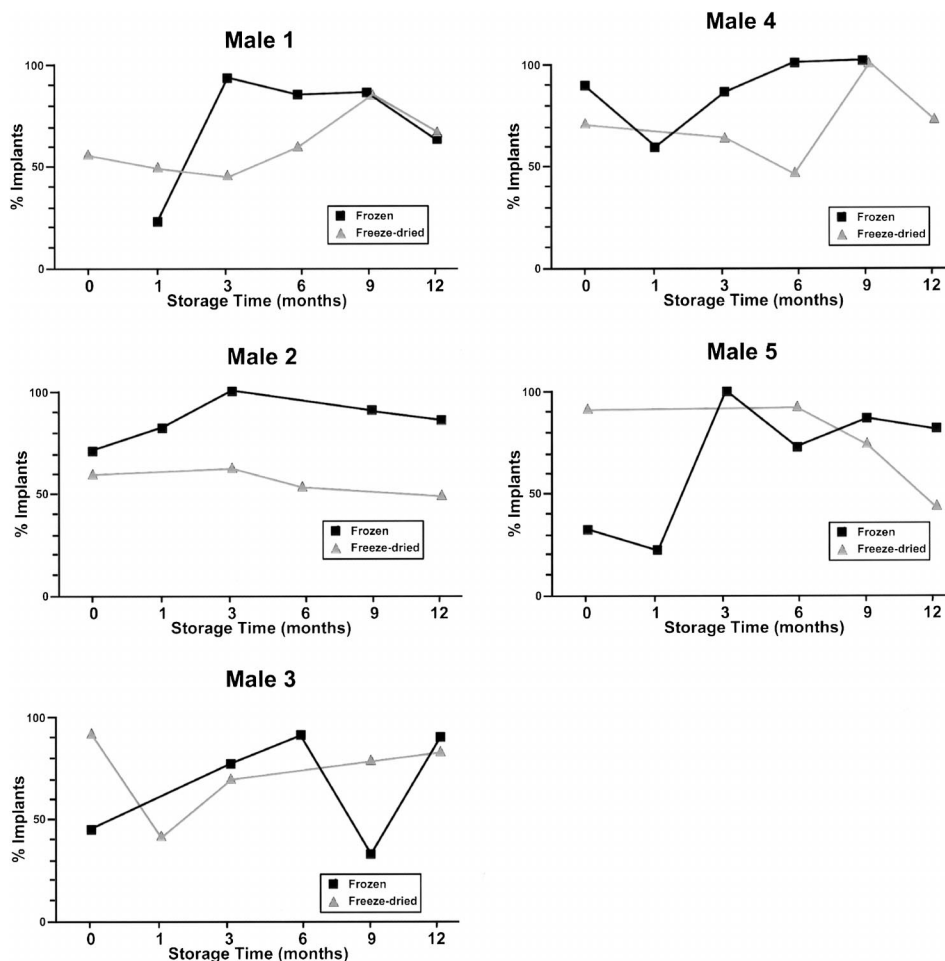


TABLE 3. Goodness-of-fit test and the estimates of the regression coefficients and their SEMs made for each of the five males examined using the Hosmer-Lemeshaw test for analysis of fetal development of embryos obtained with freeze-dried spermatozoa and with spermatozoa frozen without cryopreservation.

Mouse	Hosmer-Lemeshaw test (<i>P</i>)	Regression on time of storage			Regression on preservation method		
		b_1^a	SEM	P^b	b_2^c	SEM	P^b
1	0.148	-0.0085	0.0074	0.280	0.6064	0.3965	0.180
2	0.936	0.0836	0.0424	0.048	1.7829	0.4047	6.5E-6
3	0.113	0.0482	0.0420	0.259	0.4731	0.3756	0.280
4	0.242	0.0508	0.0503	0.325	0.9924	0.3905	0.016
5	0.132	0.1098	0.4460	0.013	0.4447	0.3927	0.344

^a Linear regression on storage time.

^b Probability that the regression coefficients are significantly different from zero.

^c Regression on preservation method.

mice. Surprisingly, four of the regressions are positive, which suggests that the yield of fetuses increases with storage time. Two of the five estimates of the regression on method are significantly different from zero (males 2 and 4), but the other three are not. However, the five estimates are marginally homogenous ($P = 0.05$), indicating that the differences between mice are not particularly great compared with the background variation ($\chi^2 = 7.895$, $df = 4$, $P = 0.096$). The weighted regression (\pm SEM) is significantly different from zero (0.8433 ± 0.1751). Pooling the data for all males, the proportion of fetuses that developed from the transferred two-cell embryos produced with freeze-dried spermatozoa (34%) was significantly lower than those produced from spermatozoa rapidly frozen without cryoprotection or with fresh spermatozoa (52% and 58%, respectively; $P < 0.001$). Fetal development was similar for fresh and rapidly frozen spermatozoa. The variability observed in Figure 2, which was more pronounced in frozen than in freeze-dried samples, may reflect the well-known phenomenon of overall variability in embryo transfer. The design of this study demanded that the experiments be performed at different times, i.e. months apart; seasonal fluctuations of diet, temperature, etc. contributed to variation in breeding performance.

Live-Born Mice Produced from Sperm Stored for 1.5 Years

Spermatozoa from two of the males (3 and 4) were freeze-dried and frozen without a cryoprotectant and injected into oocytes after 1.5 years of storage. Live-born offspring were obtained, reached maturity, and successfully reproduced. Proportions of live pups developed from transferred two-cell stage embryos were 21% (15/72) and 59% (40/68) for freeze-dried spermatozoa and those frozen without cryoprotection, respectively. Overall success rates (the proportion of live born mice developed from oocytes injected) were 18% (15/85) and 48% (40/83) for freeze-dried

spermatozoa and those frozen without cryoprotection, respectively. The proportions of live-born offspring obtained were significantly higher ($P \leq 0.001$) for spermatozoa frozen without cryoprotection than for spermatozoa that were freeze-dried. However, with both methods of preservation live, healthy, fertile progeny were obtained in sufficient numbers to establish a breeding colony for the purposes of rescuing mutant genomes of value in genetic research.

Shipment of Spermatozoa Frozen Without Cryoprotection

To examine whether spermatozoa frozen without cryoprotection and preserved in LN₂ can be shipped successfully, the integrity of sperm chromosomes was evaluated after various treatments: 1) fresh spermatozoa suspended in EGTA Tris-HCl-buffered solution (fresh control), 2) spermatozoa frozen without cryoprotection and stored for at least 1 day in LN₂, 3) spermatozoa frozen without cryoprotection and stored for at least 1 day in LN₂ followed by 48 h storage on dry ice, and 4) spermatozoa frozen without cryoprotection, stored for at least 1 day in LN₂, then for 48 h on dry ice, and then restored to LN₂ for at least 1 day. The combined data from three replicated experiments are shown in Table 4. The genetic integrity of spermatozoa was maintained under all conditions, indicating that shipment of samples on dry ice had no detrimental effect on spermatozoa rapidly frozen in LN₂ without cryoprotection.

DISCUSSION

The present data show clearly that mouse spermatozoa can retain their ability to generate viable offspring after storage for up to 1.5 years by preservation methods that are considered to be nonconventional, namely freeze-drying and rapid freezing in the absence of a cryoprotectant. Spermatozoa are no longer alive after storage by these techniques. Sperm plasma membranes are damaged and sperm

TABLE 4. Chromosome integrity of B6D2F₁ spermatozoa frozen without cryoprotection: the effect of shipment in dry ice.

Exponent	Sperm storage ^a	No. oocytes injected	No. (%) oocytes survived	No. metaphases examined	No. (%) normal metaphases ^b	Aberrations per spermatozoon ratio
1	None (fresh control)	111	97 (87)	79	75 (95)	0.05
2	LN ₂	90	79 (88)	71	69 (97)	0.03
3	LN ₂ + DI	54	46 (85)	41	40 (98)	0.03
4	LN ₂ + DI + LN ₂	109	97 (89)	91	85 (93)	0.10

^a Experiment was replicated three times (three different males). Fresh control = fresh epididymal spermatozoa suspended in EGTA Tris-HCl-buffered solution; LN₂ = samples stored in liquid nitrogen; LN₂ + DI = samples stored in LN₂ then transferred to dry ice (DI) for 2 days; LN₂ + DI + LN₂ = samples stored in LN₂, transferred to DI for 48 h, and then transferred back to LN₂.

^b No significant differences were found between treatments.

DNA becomes vulnerable when exposed to ordinary physiological solutions. The mechanism of DNA degradation is unclear but is thought to be associated with the activation of endogenous nucleases present in spermatozoa [40–42] by divalent cations such as Ca^{2+} present in culture media. Most endonucleases require calcium and/or magnesium ions and can be inhibited by divalent cation-chelating agents. We have reported previously that EDTA in Ca^{2+} -free and Mg^{2+} -free medium maintains chromosome integrity of spermatozoa better than does an ordinary medium [29, 32]. We have also found that chelating agents (EGTA and EDTA) can inhibit sperm chromosome damage induced by detergent and dithiothreitol [36] and by exogenous DNA [37]. The EGTA Tris-HCl-buffered solution used in this and other studies is not physiological in the conventional sense, but it is effective in protecting sperm DNA from degradation.

Genetic integrity of spermatozoa was evaluated by examining chromosomes of one-cell embryos after ICSI. This technique has been used routinely to evaluate sperm DNA damage [29, 35–37]. The incidence of normal karyotypes in the zygote is correlated with embryo development *in vitro* [37] and is considered a good indicator of the developmental potential of the embryo. The ICSI procedure itself does not damage sperm chromosomes [35]. There were marked differences between the two methods of sperm preservation used in this study in their ability to maintain sperm chromosome integrity. Freezing without cryoprotection did not induce significant chromosome damage compared with fresh control spermatozoa, whereas freeze-drying did. This damage may be caused by the two steps in the procedure for freeze-drying: rapid freezing followed by slow freeze-drying. Nevertheless, >50% of spermatozoa (embryos) had normal chromosomes after freeze-drying, and there was no further increase in the proportion of spermatozoa with abnormal karyotypes during storage.

The nonconventional methods of sperm preservation necessitate the use of ICSI to achieve fertilization because the spermatozoa are no longer alive after storage. Remarkably, the sperm DNA retains the capacity to form a pronucleus after injection into the oocyte, to complete the final stages of fertilization, and to generate live offspring. After preservation by both of these methods, the injected sperm head also retains the ability to activate the oocyte, indicating that the activating factor(s) [30, 31] also have been preserved. In the mouse, unlike other mammalian species such as cattle and humans, the sperm (paternal) centriole does not appear to participate in the formation of the spindle in early cleavage [43]; therefore, it is unknown whether sperm centrioles would be functional after preservation by methods used in this study.

Survival and fertilization of oocytes after ICSI with freeze-dried spermatozoa and spermatozoa rapidly frozen without cryoprotection were similar for several different inbred mouse strains (BALB/c, 129/SvJ, C57BL/6) and the B6D2F₁ hybrid [29]. At least for the mouse, ICSI is superior to IVF after spermatozoa have been cryopreserved [35]. In this study, the spermatozoa were preserved from the inbred FVB strain, which is widely used in the production of transgenic mice. Both freeze-drying and freezing without cryoprotection were effective for preserving the spermatozoa of this strain.

Comparison of the postimplantation development of embryos produced with spermatozoa that had been freeze-dried or frozen without cryoprotection revealed a striking difference between the production of normal karyotypes

and the production of implants and fetuses. The karyotypes analyses revealed few differences among male sperm donors, whereas the postimplantation analyses revealed large variation among males. The reason for this heterogeneity may be large variations in the responses of the surrogate mothers, e.g., maintaining pseudopregnancy and pregnancy blocking (Bruce effect) by the presence of strange males [44]. Large variations associated with implantation and the development of fetuses in mice are well known in teratological studies. Here, we observed this extra random variability in implantation rate that was beyond our control. Nevertheless, careful examination of individual data showed that the effect of storage time and method of preservation does not affect the proportion of transferred embryos that implanted, and thus this variability must be due to other causes. The proportion of fetuses that developed from the embryos produced with freeze-dried spermatozoa was significantly lower than that for spermatozoa frozen without cryoprotection. However, about a third of the embryos derived from freeze-dried spermatozoa developed to Day 15 of gestation. With both methods of sperm preservation, live, healthy, fertile offspring were obtained after long-term (18 mo) sperm storage. Nevertheless, the overall success rate was significantly higher with embryos derived from spermatozoa that were rapidly frozen without cryoprotection.

One of the important parameters of sperm preservation methodology is the number of samples that can be obtained from one male. We routinely disperse spermatozoa from one male in 1-ml of EGTA Tris-HCl-buffered solution. This solution is then used for freeze-drying or freezing without cryoprotection. Freeze-dried spermatozoa are stored in 2-ml glass ampules in a volume of 100 μl . Therefore, we are able to preserve 10 individual samples from one male. Sperm frozen without cryoprotection is stored in 200- μl straws, 10 μl of sperm suspension per straw. We can preserve 100 straws with spermatozoa from one male. The number of samples can be increased by dispersing spermatozoa in a higher volume of EGTA buffer prior to preservation (2 ml instead of 1 ml), but the disproportion between freeze-drying and freezing without cryoprotection remains. Thus, when many samples are needed, freezing without cryoprotection seems to be the best method.

One of the advantages of freeze-drying technology is that the sperm samples can be stored at 4°C, and shipped at ambient temperatures by regular air-mail [26]. For freezing without cryoprotection, LN₂ is needed. Although LN₂ is easily available in most facilities involved in mouse-based sciences and technologies, the problem arises when samples preserved at -196°C are to be shipped. Companies such as FedEx consider LN₂ a “dangerous product” and have several restrictions on its shipment. To address this issue, we performed an experiment mimicking shipment of spermatozoa on dry ice. Sperm samples preserved in LN₂ in EGTA Tris-HCl-buffered solution were transferred to dry ice for 48 h and then returned to -196°C. The results of chromosome analysis revealed that the genetic integrity of spermatozoa was well maintained. The transfer of cryopreserved cells from one temperature to another can be detrimental to their viability, but in spermatozoa preserved by freezing without cryoprotection, the membrane is already disrupted and viability is not an issue. The only concern is the integrity of the sperm DNA, which seems to be efficiently retained by addition of the EGTA Tris-HCl-buffered solution. Thus, it is possible to combine nonconventional sperm freezing and storage in liquid nitrogen with tempo-

rary exposure to -80°C needed for short-term transportation.

The overall conclusion from this study is that freeze-drying and freezing without cryoprotection can be successfully used for long-term preservation of mouse spermatozoa. Both methods have advantages and disadvantages, and the method used should be chosen on the basis of convenience. Freezing without cryoprotection does not induce sperm DNA damage, is quick and straightforward, does not require specialized equipment other than that for ICSI, can be carried out with limited expertise, and allows high numbers of samples per male to be obtained. Although frozen cattle and human spermatozoa have been routinely stored and transported in LN_2 for many decades, the disadvantage is that a constant supply of LN_2 is required for storage and shipment. Freeze-dried samples can be stored in a regular refrigerator and shipped at ambient temperatures. The present disadvantages of freeze-drying are that genetic (chromosome) integrity is compromised, fewer samples per male are available for storage, and expensive specialized equipment is required for freeze-drying. For both methods, freeze-drying and freezing without cryoprotection, the proportion of spermatozoa with normal undamaged chromosome complements does not change over time, i.e., up to 1 yr. Further technical studies are needed to improve the stability of sperm chromosomes during freeze-drying.

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