Journal of the American Association for Laboratory Animal Science
Copyright 2007
by the American Association for Laboratory Animal Science

Vol 46, No 3
May 2007
Pages 41–46

Production of Progeny Mice by Intracytoplasmic Sperm Injection of Repeatedly Frozen and Thawed Spermatozoa Experimental Technique

Toshihiro Aoto,* Yoshikazu Totsuka, Riichi Takahashi, and Masatsugu Ueda

Methods for generating genetically engineered mice have progressed, and the number of valuable mouse strains has increased rapidly, requiring methods for managing and maintaining these strains. Sperm cryopreservation and assisted-reproduction techniques, such as intracytoplasmic sperm injection (ICSI), can contribute greatly; however, the number of possible progeny is limited due to the finite number of cryopreserved preparations. The ability to refreeze and reuse sperm preparations would extend the utility of each cryopreserved sperm preparation. The purpose of this study was to develop a reproduction protocol involving ICSI that yielded live progeny after repeated freezing and thawing of a cryopreserved sperm preparation. We used mouse sperm subjected to repeat freezing and thawing in TYH medium for in vitro fertilization. Three inbred strains of laboratory mice—C57BL/6J, BALB/cA, and C3H/HeN—were reproduced by ICSI after reuse of a sperm preparation that had been frozen and thawed repeatedly. In particular, C57BL/6J progeny could be reproduced from spermatozoa frozen and thawed 10 times. From these results, we conclude that the reuse of cryopreserved spermatozoa can extend the opportunities for reproduction of progeny from cryopreserved sperm and can increase the utility of cryopreserved preparations as bioresources. Our results broaden management options regarding bioresource banking, particularly for mice.

Abbreviations: B6, C57BL/6J; C3H, C3H/HeN; BALB/c, BALB/cA; HEPES-CZB, CZB medium buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization

In human and mouse genome sequencing,[4,16] animal models have become an essential tool to study the biologic functions of genes in the pathogenesis and treatment of human diseases. Developing genetically engineered mice with loss or gain of gene function has increased markedly in various fields of biomedical research.[6,14] In addition, the saturation mutagenesis project has produced a large number of mutant mouse lines.[3,15] In these circumstances, the importance of maintaining the mutants securely as a genetic resource has increased, and the establishment of an effective system is imperative.

Space and cost requirements complicate maintaining a large number of mouse strains. The embryo banking system for preserving frozen embryos was established to solve these problems,[19,27] but frozen spermatozoa can be preserved more efficiently than can frozen embryos. Many more spermatozoa are produced than oocytes or embryos. The haploid spermatozoa genome is suitable for preservation as a genetic resource and is considered to be an effective alternative to embryos,[2,10,12] especially for genetically engineered mice. However, for many mouse strains (particularly C57BL/6J [B6]), spermatozoa that have been frozen and thawed yield a low efficiency rate of in vitro fertilization (IVF).[13,20] If spermatozoa of a valuable mouse strain could secure these genetic resources after being frozen and thawed, cryopreservation of spermatozoa becomes a powerful tool.

Intracytoplasmic sperm injection (ICSI) is a useful procedure in the production of progeny[5,7,28] and can solve some infertility issues due to loss of mobility by defective spermatozoa.[28] Li and colleagues[9] used ICSI to rescue a valuable mutant mouse strain from a 'last-of-line' mutant male mouse. Yanagimachi and colleagues[7] reported the possibility of using ICSI with freeze-dried spermatozoa to produce progeny. In addition, Lacham-Kaplan and colleagues[8] suggested that sperm frozen without cryoprotectants ('snap-freezing' methods) might be combined with ICSI to produce progeny.

The large number of spermatozoa typically frozen in a straw reflects its presumed use for IVF. However, current ICSI protocols use only a small portion of the frozen sperm, which must be thawed before use; typically the remainder is not refrozen and usually is discarded. If reproduction can be achieved by ICSI with spermatozoa frozen and thawed repeatedly, cryopreserved spermatozoa can be used more effectively. The repeated use of a cryopreserved spermatozoa preparation can markedly increase its value, particularly for mouse strains for which only a limited amount of sperm is available.

In this report, we describe reproduction by ICSI with repeatedly frozen and thawed B6, BALB/cA (BALB/c), and C3H/HeN (C3H) spermatozoa. The results indicate that spermatozoa preparations can be used after repeated freezing and thawing. Therefore the ICSI protocol in this report is useful in the management of mouse strain banking.

Materials and Methods

Animals. B6 mice were purchased from CLEA Japan (Tokyo, Japan). BALB/c, C3H, and CD1 mice were purchased from Charles River Laboratories Japan (Tokyo, Japan). Female B6 mice were obtained at 3 wk of age; female BALB/c and C3H mice were obtained at 8 wk of age. The mice were housed in air-conditioned rooms (23 ± 2 °C and 50% ± 5% humidity, with a 12:12-h light:dark schedule [lights on from 0500 to 1700]) and given commercial food (EF, Oriental Yeast, Tokyo, Japan). Sentinel mice were evaluated quarterly for mouse hepatitis virus, Sendai virus, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Mycoplasma* spp., *Pasteurella pneumotropica*,

Received: 7 Nov 2006. Revision requested: 30 Dec 2006. Accepted: 6 Jan 2007.
The YS Institute, Iwaso, Utsunomiya, Tochigi, Japan.
**Corresponding author. Email: ystg@ystg.co.jp*

Salmonella spp., *Aspiculuris tetraptera*, pinworms, intestinal protozoa, and ectoparasites; all animals had negative test results. All procedures for the handling and treatment of animals were reviewed and approved in advance by the Animal Care and Use Committee of The YS Institute and were conducted according to government-approved regulations.

Media. CZB medium buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES-CZB) supplemented with 0.1 mg/ml polyvinyl alcohol (30 to 50 kDa, water-soluble; Sigma-Aldrich, St Louis, MO) instead of bovine serum albumin[5,7] was used for the collection of oocytes from oviducts and the injection of spermatozoa. Oocytes were held in M2 medium after ICSI and embryo transfer.[17] After ICSI, oocytes were cultured in M16 medium[26] supplemented with 100 µM ethylenediamine tetraacetic acid (Dojindo Laboratories, Kumamoto, Japan).[1] Epididymal mouse spermatozoa were cryopreserved in TYH medium, which typically is used for IVF,[22] and R18S3 solution (ARK Resource, Kumamoto, Japan) that contained 18% D(+)-raffinose pentahydrate (w/v) and 3% skim milk (w/v) and which generally is used for the cryopreservation of mouse spermatozoa.[12] Spermatozoa were placed in HEPES-CZB supplemented with 12% (w/v) polyvinylpyrrolidone (360 kDa, Wako Pure Chemical Industries, Osaka, Japan) immediately before ICSI.

Oocyte collection. B6 mice were used at 3 to 6 wk of age; BALB/c and C3H mice were used at 8 to 12 wk of age. Oocytes were obtained from mice induced to superovulate by intraperitoneal injection of 5 IU equine chorionic gonadotropin (Nippon Zenyaku Kogyo, Fukushima, Japan), followed by intraperitoneal injection of 5 IU human chorionic gonadotropin (Sankyo Zoki, Tokyo, Japan) after 48 h. At 16 to 20 h after injection of human chorionic gonadotropin, cumulus-oocyte complexes were recovered from ampullae by use of M2 medium containing 0.1% hyaluronidase (Sigma-Aldrich). The denuded oocytes were rinsed thoroughly and maintained in M16 medium for as long as 2 h at 37 °C under 5% CO_2 in air.

Collection, freezing, and thawing of epididymal spermatozoa. Epididymal spermatozoa were obtained from each mouse strain (8 to 12 wk old). The caudal epididymides were removed from each mouse, and sperm was collected with a 25-gauge needle. The sperm collected from one male mouse was transferred to R18S3 (50 µl), and that from the other was added to TYH (300 µl). R18S3 solution containing sperm was loaded into a straw and plunged into liquid nitrogen according to the method described by Nakagata;[12] prior to use of the sperm, the straw was removed from the liquid nitrogen and the sperm thawed according to the method described by Nakagata.[12] The contents of the straw were released into a culture dish (35 mm), and 10 µl of the sperm suspension was added to TYH medium (30 to 100 µl), which is the buffer typically used for IVF.

When TYH medium was used as the freezing solution for spermatozoa, the sperm were incubated for 30 min at 37 °C under 5% CO_2 in air to allow sperm to diffuse; the resulting uniformly diffused sperm solution was divided into 20-µl aliquots, which were placed at the bottoms of 1.0-ml cryotubes (catalog no. 343958; Nunc A/S, Roskilde, Denmark). The tubes were placed rapidly directly into liquid nitrogen and stored. After 2 to 171 d, the tubes were removed from the liquid nitrogen and rapidly thawed in a 37 °C waterbath. The contents were placed in a culture dish, diluted with an equivalent volume of TYH medium (20 µl), and covered with mineral oil. Before ICSI the culture dish was kept on a plate cooled to 17 to 20 °C.

Refreezing spermatozoa. After thawing the cryopreserved spermatozoa preparation in the straw, the solution was refrozen by the same procedure as described earlier, that is, the thawed solution was diluted with TYH medium. Half of the spermatozoa suspension in the straw was transferred into a 1.0-ml cryotube, and then the tube was placed rapidly directly into liquid nitrogen and stored for 1 to 72 d until use. The spermatozoa refrozen in TYH medium again were thawed (third time overall), diluted with TYH medium, and refrozen in the same manner as previously.

ICSI. ICSI was performed according to the procedures of Kimura and Yanagimachi with some modifications.[5] Briefly, the spermatozoa were injected into oocytes at room temperature (25 to 27 °C) instead of decreased temperature (17 to 18 °C). A small quantity of spermatozoa suspension was transferred into a small volume of HEPES-CZB medium containing 12% (w/v) polyvinylpyrrolidone, and mixed well. A spermatozoon repeatedly was sucked into an injection pipette from the tail and then ejected. These procedures were repeated to remove the tail at the head-midpiece junction (neck) by piezoelectric pulses (settings: cutting, speed 7 and intensity 6; ICSI, speed 1 and intensity 1; PMAS-CT150, PrimeTech LTD, Ibaraki, Japan). The spermatozoon head then was injected into the oocytes. The injection was performed in HEPES-CZB medium within 2.5 h after thawing the frozen spermatozoa. After thawing, only spermatozoa cryopreserved with R18S3 solution had motility, and mobile spermatozoa were selected for injection, as were additional, random nonmotile spermatozoa.

Preimplantation culture. After ICSI, the oocytes were transferred to cooled M2 medium (17 to 20 °C) and kept for 15 to 40 min to maximize the survival rate of the injected oocytes.[5] They then were transferred to a drop (30 to 50 µl) of M16 medium that was equilibrated overnight with humidified 5% CO_2 in air at 37 °C. The drops on a plastic culture dish (catalog no. 351008; Falcon, Becton Dickinson, Franklin Lakes, NJ) were covered with mineral oil and cultured in humidified 5% CO_2 in air at 37 °C. The survival of ICSI-treated oocytes was examined at 6 h after culture, and the number of 2-cell stage embryos was counted at 24 h culture. Fertilized oocytes that developed to the 2-cell stage continued to be cultured, and the developmental stage of blastocysts was examined at 96 h.

Embryo transfer. Two-cell stage embryos developing from fertilized oocytes by ICSI were selected at random and transferred into oviducts of pseudopregnant CD1 female mice on day 1 of pseudopregnancy; these female mice were prepared by mating with vasectomized CD1 male mice the previous night. The female mice were euthanized after bearing offspring, and the number of implantation sites was examined.

Reproduction test. The reproduction ability of offspring obtained from embryos produced by ICSI with spermatozoa that had undergone repeated freezing and thawing (B6, 10 rounds; BALB/c and C3H, 3 rounds each) was examined. The offspring were mated with the same strain mice purchased from the same breeder (CLEA Japan or Charles River Japan) as were spermatozoa donors, and their reproduction abilities were examined.

Chromosome analysis. Offspring obtained by ICSI with fresh (control) or refrozen spermatozoa underwent chromosome analysis at the International Council for Laboratory Animal Science monitoring center (Kanagawa, Japan). Slides of metaphase chromosomes were prepared as follows: lymphocytes prepared from blood samples collected from the retrobulbar venus plexus were cultured in RPMI medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, 3 µg/ml concanavalin A, 10 µg/ml lipopolysaccharide, 55 µM 2-mercaptoethanol, and penicillin (100 U/ml)–streptomycin (100 µg/ml) (Gibco BRL, Rockville, MD) at 37 °C for 42 h. After culture with 0.02 µg/ml colcemid for 25 min, the cells were treated with hypotonic solution and methanol-acetic acid fixation, and slides were prepared.[25] The

Table 1. In vitro development of embryos produced after ICSI with C57BL/6 spermatozoa cryopreserved in R18S3 solution or TYH medium

Medium for sperm dispersion and storage	No. of oocytes (No. of experiments)	No. of oocytes that survived (%)	No. of 2-cell embryos (%)	No. of blastocysts (%)	Range of development (%) among experiments
R18S3	133 (7)	126 (94.7)	126 (100.0)	101 (80.2)	71.4–100.0
TYH	89 (7)	87 (90.8)	79 (90.8)	69 (87.3)	75.0–100.0

In all experimental groups, C573L/6 oocytes were used, and sperm had been frozen at –196 °C.
The R18S3 solution for cryopreservation of epididymal mouse spermatozoa contained 18% D (+)-raffinose pentahydrate (w/v) and 3% skim milk (w/v).
TYH is a medium for in vitro fertilization of mouse strains.

Table 2. In vivo development of C57BL/6, BALB/c, and C3H/HeN embryos derived by ICSI with spermatozoa cryopreserved in R18S3 solution or TYH medium

Medium	Mouse strain	No. of oocytes (No. of experiments)	No. of oocytes that survived (%)	No. of 2-cell embryos (%)	No. of transferred embryos	No. of recipients	Offspring production efficiency		
							No. of implants (%)	No. of offspring (%)	Range (%)
R18S3	C57BL/6	198 (6)	179 (90.4)	171 (95.5)	113	10	86 (76.1)	55 (48.7)	30.0–70.0
	BALB/c	103 (3)	93 (90.3)	90 (96.8)	81	7	57 (70.4)	24 (29.6)	7.7–50.0
	C3H/HeN	80 (3)	71 (88.8)	69 (97.2)	54	6	38 (70.4)	25 (46.3)	23.1–69.2
TYH	C57BL/6	147 (7)	141 (95.9)	135 (95.7)	112	11	94 (83.9)	72 (64.3)	53.8–75.0
	BALB/c	108 (5)	89 (82.4)	88 (98.9)	88	6	68 (77.3)	32 (36.4)	14.3–50.0
	C3H/HeN	168 (6)	151 (89.9)	142 (94.0)	101	9	70 (69.3)	44 (43.6)	37.5–60.0

In all experimental groups, oocytes used for ICSI were the same strain as the sperm, and sperm had been frozen at –196 °C. CD1 female mice were mated with vasectomized CD1 males, and 2-cell embryos were transferred to the mice on the first day of pseudopregnancy. The R18S3 medium for cryopreservation of epididymal mouse spermatozoa contained 18% D (+)-raffinose pentahydrate (w/v) and 3% skim milk (w/v). TYH is a medium for in vitro fertilization of mouse strains.

slides were air-dried and stained with 5% Giemsa solution for 50 min. At least 50 metaphases per mouse were evaluated. For karyotype analysis, slides were rinsed with distilled water and stained with 0.01 µg/ml Hoechst 33258 for 5 min and then 5.0 µg/ml quinacrine mustard for 20 min. Karyotypes were analyzed by fluorescent microscopy (DM RXA2, Leica, Heidelberg, Germany) by use of CW4000 software (Leica); 5 to 14 metaphases were examined per mouse.

Statistical analysis. Chi-square analysis and Fisher exact tests were used for statistical analysis.[7]

Results

Influence of the solution used to freeze spermatozoa. The preimplantation development of fertilized B6 oocytes produced by ICSI with B6 spermatozoa frozen in TYH medium and R18S3 solution is shown in Table 1. The percentage of embryos that developed to the blastocyst stage from the 2-cell stage was 80.2% with R18S3 solution and 87.3% with TYH medium. The developmental efficiency of embryos (the percentage of embryos that developed to blastocyst stage) generated by ICSI with refrozen spermatozoa is shown in Table 2. The birth rate after use of B6 spermatozoa frozen in TYH medium was higher than that after use of B6 spermatozoa frozen in R18S3 solution (64.3% versus 48.7%, $P \leq 0.05$). The birth rates of both BALB/c and C3H strains were equivalent between the freezing solutions of R18S3 and TYH (BALB/c, 29.6% versus 36.4%; C3H, 46.3% versus 43.6%).

Reuse of spermatozoa. The in vitro development of B6 oocytes fertilized by ICSI with repeatedly cryopreserved B6 spermatozoa is shown in Table 3. The developmental efficiency of spermatozoa initially frozen in R18S3 decreased according to the frequency of refreezing (70.2% to 55.4%); however, spermatozoa initially frozen in TYH showed a stable value after repeated freezing with TYH (88.5% to 87.3%). The in vivo development of 3 inbred mouse strains from oocytes fertilized with repeatedly cryopreserved spermatozoa by ICSI is shown in Table 4. The developmental efficiency after use of B6 spermatozoa initially frozen in R18S3 solution and refrozen in TYH medium was lower than that after use of spermatozoa repeatedly frozen only in TYH medium (30.8% versus 63.7%, $P \leq 0.05$). The efficiency of in vivo development after use of B6 spermatozoa frozen initially in TYH medium decreased slightly according to the increase in the number of recryopreservation cycles (63.7% versus 48.5%, $P \leq 0.05$) (Table 4). BALB/c and C3H spermatozoa also produced live young after repeated freezing in TYH medium (Table 4). Approximately 20% of the 2-cell embryos produced with BALB/c spermatozoa and 40% to 60% of those produced with C3H spermatozoa after repeated cryopreservation and ICSI developed into live progeny.

Reproductive ability and karyotypes of mice produced by ICSI with refrozen spermatozoa. The fertility of B6, BALB/c, and C3H offspring generated by ICSI with repeatedly cryopreserved spermatozoa was examined (Table 5). Almost all offspring were fertile; however, 2 (both male) of 30 B6 mice (17 male and 13 female) produced by ICSI with spermatozoa frozen and thawed 10 times were infertile, and 2 of 29 mice had an abnormal karyotype; 1 female offspring examined for fertility died for no apparent reason before chromosomal analysis (Table 5). In comparison, the 10 control pups (offspring generated by ICSI with fresh B6 spermatozoa) examined all had normal karyotypes. The karyotypes of offspring from BALB/c and C3H were not examined because of their normal fertility.

Discussion

Two challenges inherent to the management of mouse colonies are particularly problematic in regard to genetically engineered mice. One is the limited breeding space available for expanding the many diverse strains;[6] the other is altered reproductive ability due to gene modification.[9] To preserve these valuable bioresources, the efficacy of their use must be improved.

Regarding banking of genetically engineered strains, it is easier and more economical to cryopreserve spermatozoa than of eggs and embryos. Excellent procedures for IVF and ICSI have been reported and successfully generate living offspring from frozen

Table 3. In vitro development of embryos produced after ICSI with C57BL/6 spermatozoa that underwent repeated cryopreservation in TYH medium

Medium for sperm dispersion and storage (Reiterating treatment)	No. of oocytes (No. of experiments)	No. of oocytes that survived (%)	No. of 2-cell embryos (%)	No. of blastocysts (%)	Range of development (%) among experiments
R18S3→TYH	63 (3)	60 (95.2)	57 (95.0)	40 (70.2)	60.0–80.0
TYH→TYH	64 (3)	63 (98.4)	61 (96.8)	54 (88.5)	80.8–94.7
R18S3→TYH→TYH	84 (4)	78 (92.9)	74 (94.9)	41 (55.4)	50.0–66.7
TYH→TYH→TYH	77 (4)	74 (96.1)	71 (95.9)	62 (87.3)	75.0–95.2

In all experimental groups, C57BL/6 oocytes were used, and sperm were refrozen at –196 °C.
The R18S3 medium for cryopreservation of epididymal mouse spermatozoa contained 18% D (+)-raffinose pentahydrate (w/v) and 3% skim milk (w/v). TYH is a medium for in vitro fertilization of mouse strains.

Table 4. In vivo development of C57BL/6J, BALB/cA, and C3H/HeN embryos generated by ICSI using spermatozoa repeatedly frozen in TYH medium

Sperm treatment	Strain	No. of oocytes (No. of experiments)	No. of oocytes that survived (%)	No. of 2-cell embryos (%)	No. of embryos transferred	No. of recipients	Offspring production efficiency		
							No. of embryos implanted (%)	No. of offspring (%)	Range (%)
R18S3→TYH	C57BL/6J	170 (4)	159 (93.9)	149 (93.7)	120	9	73 (60.8)	37 (30.8)	15.4–53.8
TYH→TYH	C57BL/6J	113 (4)	100 (88.5)	98 (98.0)	91	8	80 (87.9)	58 (63.7)	38.5–84.6
R18S3→TYH→TYH	C57BL/6J	206 (4)	194 (94.2)	170 (87.6)	102	8	61 (59.8)	32 (31.4)	8.3–50.0
	BALB/cA	87 (5)	78 (89.7)	71 (91.0)	69	5	41 (59.4)	14 (20.3)	7.7–28.6
	C3H/HeN	22 (2)	20 (90.9)	20 (100.0)	20	2	18 (90.0)	8 (40.0)	37.5-41.7
TYH→TYH→TYH	C57BL/6J	110 (3)	106 (96.4)	101 (95.3)	99	8	83 (83.8)	48 (48.5)	33.3–66.7
	BALB/cA	116 (4)	97 (83.6)	88 (90.7)	82	6	54 (65.9)	20 (24.4)	12.5–66.7
	C3H/HeN	67 (3)	59 (88.1)	58 (98.3)	38	3	35 (92.1)	23 (60.5)	53.8–64.7

In all experimental groups, oocytes used for ICSI were from the same strain as that of spermatozoa, which were refrozen at –196 °C. CD1 female mice were mated with vasectomized CD1 male mice, and 2-cell embryos were transferred to the mice on the first day of pseudopregnancy.
The R18S3 medium for cryopreservation of epididymal mouse spermatozoa contained 18% D (+)-raffinose pentahydrate (w/v) and 3% skim milk (w/v). TYH is a medium for in vitro fertilization of mouse strains.

spermatozoa.[28] Numerous living offspring can be obtained by IVF with cryopreserved spermatozoa, providing the spermatozoa retain motility and fertility. Although spermatozoa often lose motility after freezing, excellent ICSI procedures can generate living offspring from nonmotile spermatozoa.[28] We therefore focused on ICSI and cryopreservation of spermatozoa for the management of valuable genetically engineered mouse strains.

Typically only a small portion of the large number of spermatozoa frozen in a straw are used after thawing; the remainder usually is discarded. Successful methods for using refrozen spermatozoa with ICSI to generate progeny would greatly increase the utility of these bioresources. We first examined the generation of offspring by ICSI with spermatozoa frozen in R18S3 solution, a typical buffer for cryopreservation of mouse spermatozoa,[12] and TYH medium, which generally is used for IVF.[22] We successfully generated live B6, BALB/c, and C3H progeny from spermatozoa frozen in either media (Table 2). In current IVF protocols, spermatozoa typically are cryopreserved in R18S3 solution;[12,13] therefore we evaluated the generation of offspring by ICSI with spermatozoa refrozen in TYH medium after initial freezing in R18S3 solution or TYH medium. Regardless of initial freezing buffer used, ICSI with refrozen spermatozoa yielded living B6, BALB/c, and C3H progeny (Table 4). We were unable to define a clear reason for the more favorable results with TYH medium, but the experience of Yanagimachi and colleagues[11] may provide some insight. Those investigators reported that although removal of sperm plasma membrane and acrosome was not a prerequisite for producing offspring by ICSI, doing so resulted in earlier onset of oocyte activation and better embryonic development. In our study the TYH medium without cryoprotection that we used to freeze spermatozoa might have created favorable conditions for embryonic development. According to our results, refrozen spermatozoa can be a bioresource for preserving valuable mouse strains.

Moreover, we examined the reproductive ability of the progeny produced by ICSI of refrozen spermatozoa. Although 2 of 30 B6 offspring generated from spermatozoa that had been frozen and thawed were infertile and had an abnormal karyotype, offspring with normal fertility had normal karyotypes, as did control offspring (Table 5). Successful reproduction of mice by ICSI with spermatozoa frozen without cryoprotectants has been reported,[8,23] and the genetic integrity of embryos produced from frozen spermatozoa should be examined.[7,21,24] In addition, abnormalities in the chromosomes of human spermatozoa increased 10-fold after 2 cycles of freezing and thawing.[18] In comparison we found chromosome aberration only in offspring that showed reproductive disturbance, and those having normal reproduction ability maintained a normal karyotype. Therefore, accumulation of aberrations seems to substantiate decreased breeding vigor. Offspring could be generated by ICSI with spermatozoa cryopreserved in TYH medium for 1 y in our preliminary experiment (data not shown). Further Ward and colleagues[24] reported that offspring could be produced by ICSI with spermatozoa after 1.5 y of cryopreservation without cryoprotectant, and this prolonged storage time had no adverse effects on genetic integrity.

In conclusion, the use of repeatedly frozen spermatozoa for the reproduction of laboratory animals by ICSI facilitates management of a mouse bioresources bank, including reduction of the number of nitrogen storage tanks needed for cryopreserva-

Table 5. Evaluation of the fertility and chromosomes of C57BL/6J, BALB/cA, and C3H/HeN mice generated by ICSI with spermatozoa repeatedly frozen in TYH medium

Strain	Sperm treatment	No. of offspring examined	No. infertile	No. with abnormal karyotype/ total no. evaluated
C57BL/6J	Frozen and thawed 10 times	30	2	2/29[a]
C57BL/6J	None (fresh)	10	Not examined	0/10
BALB/cA	Frozen and thawed 3 times	23	0	Not examined
C3H/HeN	Frozen and thawed 3 times	28	0	Not examined

Sperm were cryopreserved in liquid nitrogen (–196 °C).
When progeny were not obtained by natural mating, the mouse was classified as infertile.
[a]One female offspring examined for fertility died without apparent reason. The 2 abnormal karyotypes obtained were 40,XY,+10,–15 and 40,XY,del(3).

tion. Our success in reusing previously frozen sperm illustrates the possibility of developing a practical bioresource from spermatozoa preparations currently discarded after IVF and ICSI. Furthermore, it is possible to maintain this bioresource by repeatedly refreezing it, particularly if the frozen sample is the last one of a valuable mouse strain.

Acknowledgments

We thank T Shiroishi (Mammalian Genetics Laboratory, National Institute of Genetics, Shizuoka, Japan) for his valuable advice on the experimental design and A Ogura (RIKEN Bioresource Center, Ibaraki, Japan) for his useful advice on ICSI technology. We thank the International Council for Laboratory Animal Science Monitoring Center for karyotype analysis. We also thank our laboratory staff for their support of this study.

References

1. **Abramczuk J, Solter D, Koprowski H.** 1977. The beneficial effect EDTA on development of mouse one-cell embryos in chemically defined medium. Dev Biol **61:**378–383.
2. **Critser JK, Mobraaten LE.** 2000. Cryopreservation of murine spermatozoa. ILAR J **41:**197–206.
3. **Hrabe de Angelis MH, Flaswinkel H, Fuchs H, Rathkolb B, Soewarto D, Marschall S, Heffner S, Pargent W, Wuensch K, Jung M, Reis A, Richter T, Alessandrini F, Jakob T, Fuchs E, Kolb H, Kremmer E, Schaeble K, Rollinski B, Roscher A, Peters C, Meitinger T, Strom T, Steckler T, Holsboer F, Klopstock T, Gekeler F, Schindewolf C, Jung T, Avraham K, Behrendt H, Ring J, Zimmer A, Schughart K, Pfeffer K, Wolf E, Balling R.** 2000. Genome-wide, large-scale production of mutant mice by ENU mutagenesis. Nat Genet **25:**444–447.
4. **International Human Genome Sequencing Consortium.** 2004. Finishing the euchromatic sequence of the human genome. Nature **431:**931–945.
5. **Kimura Y, Yanagimachi R.** 1995. Intracytoplasmic sperm injection in the mouse. Biol Reprod **52:**709–720.
6. **Knight J, Abbott A.** 2002. Full house. Nature **417:**785–786.
7. **Kusakabe H, Szczygiel MA, Whittingham DG, Yanagimachi R.** 2001. Inaugural article: maintenance of genetic intergrity in frozen and freeze-dried mouse spermatozoa. Proc Natl Acad Sci U S A **98:**13501–13506.
8. **Lacham-Kaplan O, Shaw J, Sanchez-Partida LG, Trounson A.** 2003. Oocyte activation after intracytoplasmic injection with sperm frozen without cryoprotectants results in live offspring from inbred and hybrid mouse strains. Biol Reprod **69**: 1683–1689.
9. **Li MW, McGinnis L, Zhu L, Lawitts J, Biggers J, Lloyd KC.** 2003. Intracytoplasmic sperm injection (ICSI) enables rescue of valuable mutant mouse strains. Comp Med **53:**265–269.
10. **Marschall S, Hrabe de Angelis M.** 1999. Cryopreservation of mouse spermatozoa: double your mouse space. Trends Genet **15:**128–131.
11. **Morozumi K, Shikano T, Miyazaki S, Yanagimachi R.** 2006. Simultaneous removal of sperm plasma membrane and acrosome before intracytoplasmic sperm injection improves oocyte activation/embryonic development. Proc Natl Acad Sci U S A **103:**17661–17666.
12. **Nakagata N.** 2000. Cryopreservation of mouse spermatozoa. Mamm Genome **11:**572–576.
13. **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.
14. **Nolan PM.** 2000. Generation of mouse mutants as a tool for functional genomics. Pharmacogenomics **1:**243–255.
15. **Nolan PM, Peters J, Strivens M, Rogers D, Hagan J, Spurr N, Gray IC, Vizor L, Brooker D, Whitehill E, Washbourne R, Hough T, Greenaway S, Hewitt M, Liu X, McCormack S, Pickford K, Selley R, Wells C, Tymowska-Lalanne Z, Roby P, Glenister P, Thornton C, Thaung C, Stevenson JA, Arkell R, Mburu P, Hardisty R, Kiernan A, Erven A, Steel KP, Voegeling S, Guenet JL, Nickols C, Sadri R, Nasse M, Isaacs A, Davies K, Browne M, Fisher EM, Martin J, Rastan S, Brown SD, Hunter J.** 2000. A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. Nat Genet **25:**440–443.
16. **Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, Kondo S, Nikaido I, Osato N, Saito R, Suzuki H, Yamanaka I, Kiyosawa H, Yagi K, Tomaru Y, Hasegawa Y, Nogami A, Schonbach C, Gojobori T, Baldarelli R, Hill DP, Bult C, Hume DA, Quackenbush J, Schriml LM, Kanapin A, Matsuda H, Batalov S, Beisel KW, Blake JA, Bradt D, Brusic V, Chothia C, Corbani LE, Cousins S, Dalla E, Dragani TA, Fletcher CF, Forrest A, Frazer KS, Gaasterland T, Gariboldi M, Gissi C, Godzik A, Gough J, Grimmond S, Gustincich S, Hirokawa N, Jackson IJ, Jarvis ED, Kanai A, Kawaji H, Kawasawa Y, Kedzierski RM, King BL, Konagaya A, Kurochkin IV, Lee Y, Lenhard B, Lyons PA, Maglott DR, Maltais L, Marchionni L, McKenzie L, Miki H, Nagashima T, Numata K, Okido T, Pavan WJ, Pertea G, Pesole G, Petrovsky N, Pillai R, Pontius JU, Qi D, Ramachandran S, Ravasi T, Reed JC, Reed DJ, Reid J, Ring BZ, Ringwald M, Sandelin A, Schneider C, Semple CA, Setou M, Shimada K, Sultana R, Takenaka Y, Taylor MS, Teasdale RD, Tomita M, Verardo R, Wagner L, Wahlestedt C, Wang Y, Watanabe Y, Wells C, Wilming LG, Wynshaw-Boris A, Yanagisawa M, Yang I, Yang L, Yuan Z, Zavolan M, Zhu Y, Zimmer A, Carninci P, Hayatsu N, Hirozane-Kishikawa T, Konno H, Nakamura M, Sakazume N, Sato K, Shiraki T, Waki K, Kawai J, Aizawa K, Arakawa T, Fukuda S, Hara A, Hashizume W, Imotani K, Ishii Y, Itoh M, Kagawa I, Miyazaki A, Sakai K, Sasaki D, Shibata K, Shinagawa A, Yasunishi A, Yoshino M, Waterston R, Lander ES, Rogers J, Birney E, Hayashizaki Y; FANTOM Consortium; RIKEN Genome Exploration Research Group Phase I & II Team.** 2002. Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature **420:**563–573.
17. **Quinn P, Barros C, Whittingham DG.** 1982. Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. J Reprod Fertil **66:**161–168.
18. **Rybouchkin A, Dozortsev D, Pelinck MJ, De Sutter P, Dhont M.** 1996. Analysis of the oocyte activating capacity and chromosomal complement of round-headed human spermatozoa by their injection into mouse oocytes. Hum Reprod **11:**2170–2175.
19. **Shaw JM, Nakagata N.** 2002. Cryopreservation of transgenic mouse lines. Methods Mol Biol **180:**207–228.
20. **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.

21. **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.
22. **Toyoda Y, Azuma S, Itagaki Y, Hoshi T.** 1971. Studies on the fertilization of mouse eggs in vitro fertilization of eggs by fresh epididymal sperm. Jpn J Anim Reprod **16:**147–152.
23. **Wakayama T, Whittingham DG, Yanagimachi R.** 1998. Production of normal offspring from mouse oocytes injected with spermatozoa cryopreserved with or without cryoprotection. J Reprod Fertil **112:**11–17.
24. **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.
25. **Watt JL, Stephen GS.** 1986. A practical approach. In: Rooney DE, Czepulkowski BH, editors. Human cytogenetics. Oxford: IRL. p 47–49.
26. **Whittingham DG.** 1971. Culture of mouse ova. J Reprod Fertil Suppl **14:**7–21.
27. **Whittingham DG, Leibo SP, Mazur P.** 1972. Survival of mouse embryos frozen to –196 degrees and –269 degrees C. Science **178:**411–414.
28. **Yanagimachi R.** 2001. Gamete manipulation for development: new methods for conception. Reprod Fertil Dev **13:**3–14.