

# Development of normal mice from oocytes injected with freeze-dried spermatozoa

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**Freeze-dried mouse spermatozoa are all motionless and dead in the conventional sense. When injected into oocytes, however, their nuclei can support normal embryonic development even after three month preservation in a dried state. Although the freeze-drying protocol reported here will need further improvement, the results suggest that it may be possible to store the male genomes at room temperature.**

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Sperm cryopreservation<sup>1,2</sup> has revolutionized animal husbandry as well as reproductive medicine. Freeze-thawed spermatozoa can often regain their motility and be used for fertilization. Long-term storage of spermatozoa in liquid nitrogen (-196°C) has been used routinely for cattle and humans<sup>3</sup>; however, this requires a constant replacement supply of liquid nitrogen. Furthermore, liquid nitrogen (and even dry ice) may not be readily accessible in some parts of the world. Thus, it would be ideal to be able to store spermatozoa indefinitely at ambient temperatures, and many attempts have been made to achieve this. Polge et al.<sup>4</sup> first attempted freeze-drying spermatozoa by mixing 1 ml of fowl semen with an equal volume of Ringer's solution containing 20–30% glycerol, spreading it as a thin layer in a distillation flask, and freeze-drying while removing 90% of water. In preparations rehydrated within 2 h after being brought back to room temperature, up to 50% of spermatozoa regained motility, but their fertility was never determined. Subsequent attempts to freeze-dry human and bull spermatozoa in a viable state failed<sup>5,6</sup>, although a few exceptions were claimed. Yushchenko<sup>7</sup> reported the birth of 12 litters of normal rabbits using freeze-dried spermatozoa, but Saacke and Almquist<sup>8</sup> failed to duplicate the work. Meryman<sup>9</sup> and Graham and associates<sup>10,11</sup> reported that cows became pregnant after artificial insemination with freeze-dried spermatozoa, but this has not been confirmed. These results are in marked contrast to the largely successful long-term storage of viruses, bacteria, yeasts, and sporulating fungi by freeze-drying<sup>12</sup>.

We demonstrate that mouse spermatozoa can be freeze-dried without losing their genetic and reproductive potential. Although they are all dead in the conventional sense, they can support normal development when injected directly into oocytes.

## Results

When caudae epididymal spermatozoa were freeze-dried, kept in vacuum-sealed ampules (Fig. 1), and then rehydrated, none were motile. All of over 10,000 spermatozoa from four males were diagnosed as dead by live/dead cell staining. Heads and tails of some spermatozoa were separated (Fig. 2A). Unlike fresh, live spermatozoa (Fig. 2B), freeze-dried spermatozoa were lacking (or largely missing) in the plasma membrane and in the principal segment of the acrosome (Fig. 2C). Tail-intact spermatozoa were selected and their heads were separated from tails by applying a piezopulse to the neck region. Only the heads were injected individually into oocytes. The majority of the oocytes survived the operation. Most of these were activated and fertilized normally regardless of the original sperm-suspension medium (CZB<sup>13,14</sup> or

DMEM), storage temperature, and storage periods (Table 1). The majority of fertilized eggs developed into morulae/blastocysts *in vitro* and many developed into normal offspring when transferred to foster mothers (Table 1). All offspring grew normally (Fig. 3). Their sex ratio was about 1:1. Two fully grown females and two males in each of 12 experimental groups (Table 1) were randomly selected and mated. All proved to be fertile and had litters of normal size (8 to 12). The maximum storage period of ampules in this study was 3 months at 4°C. In three experiments, 57 oocytes from three females were injected with 3 month-stored spermatozoa. 95% of the injected oocytes survived operation, and all of them were fertilized normally. Ninety-one percent of fertilized eggs developed to morulae/blastocysts *in vitro*. Fourteen (30%) of 46 embryos transferred to three foster mothers developed into normal adults.

To find out whether freeze-dried spermatozoa can be shipped abroad, one of us (R.Y.) carried a few ampules during a 3-week trip to Japan (October–November, 1997). Before freeze-drying, these epididymal spermatozoa were suspended in CZB medium. No special precautions were taken except that the ampules were wrapped with aluminum foil and kept in a cardboard box the entire time. The temperature varied between 5°C and 30°C during this trip. One week after their return to Honolulu, spermatozoa were rehydrated, and 29 randomly selected spermatozoa were individually injected into oocytes. Twenty-three oocytes (79%) survived and were fertilized normally. Nineteen (83%) developed into morulae/blastocysts *in vitro*. Three of those (16%) reached full term after transfer to a foster mother. All three (two females and one male) grew into fertile adults.

## Discussion

Spermatozoa do not need to be alive in the conventional sense to support normal embryonic development. Goto et al.<sup>15</sup> reported the birth of two normal calves after injection of spermatozoa killed by freeze-thawing without cryoprotectants. In human intracytoplasmic sperm injection (ICSI), immediately before injection spermatozoa are immobilized (killed) by aggressive abrasion of tails<sup>16</sup>. In fact, sperm immobilization significantly increases the rate of ICSI success<sup>17,18</sup>. This could perhaps be due to quicker disintegration of sperm plasma membrane, and therefore quicker intermingling of the sperm nucleus with the oocyte's cytoplasm<sup>19</sup>. When mouse spermatozoa were suspended in media without any cryoprotectant, then plunged into liquid nitrogen, all were dead as judged by live/dead cell staining, and yet normal embryonic development occurred after injection of their heads<sup>20</sup>.

**RESEARCH**

Obviously, cell viability and nuclear viability are not synonymous. Although freeze-dried hamster and human sperm heads (nuclei) injected into oocytes can form normal-looking pronuclei<sup>25,26</sup>, it has never been determined that they can support normal development. It is now clear that mouse spermatozoa can retain their genetic integrity after freeze-drying. The freeze-drying and storage conditions we used

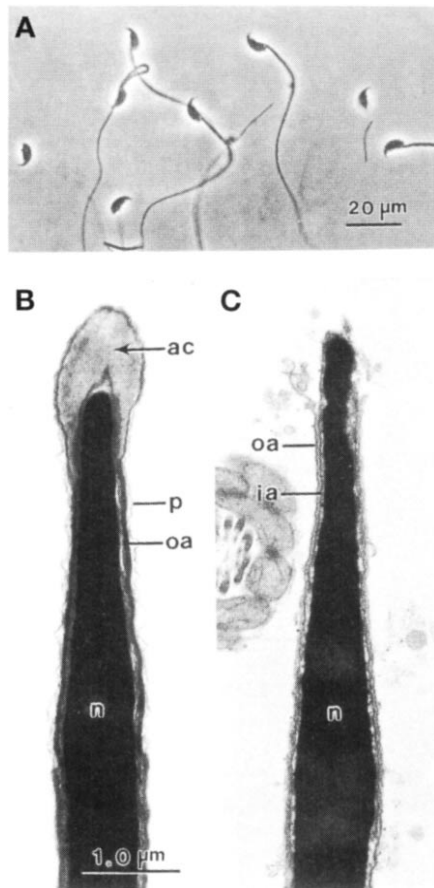
in this study are perhaps far from ideal. Sperm-suspending medium prior to freezing, freezing protocols, and the storage conditions of dried spermatozoa may need further refinement to minimize any possibility of nuclear damage. We noticed that the incidence of oocyte activation and normal fertilization following sperm injection decreased with increasing time after sperm rehydration. Rehydration medium and procedure also need to be improved.



**Figure 1.** Vacuum-sealed ampoules with freeze-dried mouse spermatozoa. The white powder at the bottom of each ampoule is the dried CZB medium containing spermatozoa.



**Figure 3.** Offspring with a CD-1 (albino) foster mother. These three young developed from B6D2F1 oocytes injected with B6D2F1 spermatozoa that had been kept at room temperature for 1 month after freeze-drying.



**Figure 2.** (A) Freeze-dried mouse spermatozoa immediately after rehydration. (B) A longitudinal section through the anterior region of unfrozen sperm head. (C) The same as B, but of a freeze-dried sperm. (p) plasma membrane, (ac) acrosome, (ia) inner acrosomal membrane, and (n) nucleus.

It is interesting that the majority of the oocytes that survived the injection of freeze-dried sperm heads were activated and fertilized normally (Table 1). Perhaps oocyte activation was induced by sperm-borne oocyte-activating molecules<sup>27,28</sup> rather than by gamete membrane ligand-receptor interactions<sup>29</sup>. It seems likely these oocyte-activating molecules are resistant to freeze-drying.

Sperm cryopreservation has been very successful in human and some farm animals<sup>5</sup>. It also has been used as a means of maintaining large number of transgenic mouse strains<sup>30</sup>. Conventional sperm preservation is very expensive in the long term because of the need for a constant supply of liquid nitrogen. Maintaining rapidly increasing numbers of transgenic mouse strains is becoming more expensive every year. Though direct sperm injection requires a trained operator and special equipment, if we can store spermatozoa in a freeze-dried state at ambient temperatures or in ordinary refrigerators, the reduction in the maintenance and shipping costs of spermatozoa would be immense.

There are many species whose spermatozoa cannot tolerate conventional freezing protocols<sup>5</sup>. Because the spermatozoa do not need to be alive to produce normal offspring, freezing without cryoprotectant<sup>24</sup> (or freeze-drying) may become an alternate method to preserve male genomes. It

**Table 1.** Development of mouse oocyte injected with freeze-dried spermatozoa.

Sperm-suspending medium	Sample storage		Sperm-injected oocytes				No. embryos transferred (No. foster females)	No. (overall %) [range]* of live offspring
	Temp. (°C)	Periods	Total no. (Exp. no.)	No. (%) surviving	No. (%) fertilized normally	No. (%) reaching morula/blastocyst stage		
CZB	25°	1 day	155 (5)	135 (87)	133 (99) <sup>a</sup>	120 (90) <sup>a</sup>	116 (8)	34 (29) [0–71]
		2 wk	158 (4)	144 (91)	141 (98) <sup>a</sup>	131 (93) <sup>f</sup>	126 (8)	25 (20) [0–30] <sup>a</sup>
		1 mo	130 (4)	120 (92)	117 (98) <sup>a</sup>	89 (76) <sup>g</sup>	87 (5)	16 (18) [5–33] <sup>a</sup>
	4	2 wk	137 (4)	126 (92)	123 (98) <sup>a</sup>	114 (93) <sup>g</sup>	105 (7)	27 (26) [0–45]
		1 mo	131 (4)	117 (89)	112 (96) <sup>a</sup>	98 (88) <sup>h</sup>	96 (6)	32 (33) [14–65] <sup>l</sup>
		3 mo	40 (2)	38 (95)	38 (100) <sup>a</sup>	35 (92) <sup>h</sup>	32 (2)	9 (28) [26–31]
DMEM	25	1 day	128 (3)	116 (91)	104 (90) <sup>b</sup>	95 (91) <sup>a</sup>	95 (6)	29 (31) [18–47]
		2 wk	109 (2)	102 (94)	85 (83) <sup>c</sup>	67 (79) <sup>h</sup>	60 (3)	8 (13) [0–20] <sup>m</sup>
		1 mo	113 (2)	106 (94)	74 (70) <sup>d</sup>	47 (64)	47 (3)	8 (17) [0–24]
	4	2 wk	83 (2)	79 (95)	79 (100) <sup>a</sup>	69 (87) <sup>h</sup>	56 (3)	20 (36) [21–46] <sup>l</sup>
		1 mo	152 (3)	137 (90)	135 (99) <sup>a</sup>	114 (84) <sup>h</sup>	54 (3)	22 (41) [15–60] <sup>n</sup>
		3 mo	17 (1)	16 (94)	16 (100)	14 (88)	14 (1)	5 (35) [35–35]

a vs. c, d; b vs. d:  $p < 0.005$ ; e vs. g, j; f vs. g, i, j; h vs. j:  $p < 0.005$ ; n vs. k, m; l vs. m:  $p < 0.01$ . \*Range of % offspring per foster mother.

is important to note that in eutherian mammals sperm nuclei are stabilized by extensive S-S crosslinking of nuclear protamines during epididymal maturation<sup>31</sup>. Perhaps this unique feature makes the nuclei of mature mouse spermatozoa resistant to such harsh physical stresses as freezing without cryoprotection and freeze-drying.

### Experimental protocol

**Animals.** Animals used in this study were maintained in accordance with the guidelines of the Laboratory Animal Service at the University of Hawaii and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council (DHEW publication no. [NIH] 80-23, revised in 1985). The protocol of our animal handling and treatment was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii.

**Sperm collection and freeze-drying.** For each experiment, two caudae epididymides of a mature B6D2F1 male were used. While applying finger pressure to each epididymis, its distal portion was punctured with sharp forceps. The dense sperm that subsequently oozed from the epididymis was transferred into a 1.5 ml polypropylene tube containing 1 ml of a test medium. Two major test solutions were CZB medium<sup>17,18</sup> without EDTA and DMEM (Sigma, St. Louis, MO) supplemented with 10% (vol/vol) fetal bovine serum (Hyclone; Logan, UT). After incubation for 30 min at 37.5°C, the upper 0.3–0.5 ml of the sperm suspension was removed from the tube. Over 90% of spermatozoa in this suspension (approximately 3–10 × 10<sup>6</sup>/ml) were actively motile. An aliquot (100 µl) of the sperm suspension was put in a 2 ml ampule (Wheaton Scientific, Millville, NJ), which was plunged directly into liquid nitrogen. Ten minutes later, ampules were placed in a precooled (-50°C) freeze-flask attached to a freeze-dry system (Model 10-020; VirTis, Gardner, NY). Inlet pressure was approximately 1 mtorr. About 12 h later, the flask was removed from the system after it had been filled with argon supplied by way of a gas-drying jar (Fisher Scientific, Pittsburgh, PA). Each ampule was connected to a vacuum pump and frame-sealed after >99% of the gas was pumped out of it. Ampules were individually wrapped with aluminum foil and stored at room temperature (about 25°C) or at 4°C. In preliminary experiments we tested simpler media to suspend spermatozoa prior to freeze-drying. Spermatozoa were first suspended in CZB medium, centrifuged, resuspended in one of the following media, and freeze-dried immediately: (1) distilled water, (2) 34% sucrose in distilled water, (3) 180 mg/ml raffinose plus 5 mg/ml BSA, (4) 0.9% NaCl with 5 mg/ml bovine serum albumin, (5) 0.9% NaCl with 1 mg/ml glucose plus 5 mg/ml BSA, and (6) EDTA-, lactate- and calcium-free CZB. As we found that only EDTA-, lactate- and calcium-free CZB was as good as the regular CZB in its ability to keep sperm nuclei development-competent after freeze-drying, we decided to use only regular CZB and DMEM throughout the study.

**Rehydration.** After breaking an ampule, 100 µl of distilled water was added to the dried spermatozoa. Then 5 µl of sperm suspension was thoroughly mixed with 50 µl Hepes-CZB containing 12% (wt/vol) polyvinylpyrrolidone (average molecular weight 360 kDa; Sigma). Sperm viability was assessed by using a commercially available cell viability test kit (Live/dead FertiLight; Molecular Probes, Eugene, OR) which differentiates between plasma membrane-intact (live) and -damaged (dead) cells according to fluorescence staining pattern under an ultraviolet microscope. The nuclei of live spermatozoa fluoresced green whereas those of dead spermatozoa fluoresced bright orange-red.

**Oocyte preparation and sperm injection.** Mature B6D2F1 females were induced to superovulate by consecutive injections of pregnant mare serum gonadotropin and human chorionic gonadotropin (hCG) 48 h apart. Fourteen hours after hCG injection, cumulus-oocyte complexes were collected from oviducts and treated with bovine testicular hyaluronidase (300 USP units/ml) in Hepes-buffered CZB medium (Hepes-CZB)<sup>12</sup> for 3 min to disperse cumulus cells. Before sperm injection the oocytes were rinsed and stored in CZB medium for up to 4 h at 37.5°C in an atmosphere of 5% CO<sub>2</sub> in air. A single spermatozoon was aspirated into an injection pipette attached to a Piezo electric pipette-driving unit<sup>32</sup>. After the sperm head and tails were separated by applying a single or a few piezopulses to the neck region, each head alone was injected into each oocyte. All injections were performed in Hepes-CZB at room temperature within 1 h of sperm rehydration. Sperm-injected oocytes were incubated in CZB at 37.5°C under 5% CO<sub>2</sub> in air and examined 5–6 h later. Those with two distinct pronuclei and a second polar body were considered normally fertilized.

**Embryo transfer.** Normal fertilized eggs were cultured for 4 days and those reaching the morula or blastocyst stage were transferred into the uterine horns of recipient CD-1 females (albino) that had been mated with vasectomized CD-1 males 3 days previously. A mean number of eight morulae/blastocysts was trans-

ferred into each horn. Females were allowed to deliver and raise their surrogate offspring (which had black, brown, or gray coats). Some mature male and female offspring were randomly selected and mated to examine their fertility.

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