Production of normal offspring from mouse oocytes injected with spermatozoa cryopreserved with or without cryoprotection

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Epididymal mouse spermatozoa were suspended in various physiological solutions (CZB, PBS or isotonic saline) with or without 18% (w/v) raffinose before cooling to −20°, −50° or −196°C and storage for 1–28 days. After thawing, a few spermatozoa frozen with raffinose were partially motile (about 2%) but in all other treatments they were immotile and diagnosed as ‘dead’ by staining that differentiates between live and dead spermatozoa. Almost all oocytes injected with sperm heads (nuclei) from spermatozoa frozen with and without raffinose were fertilized normally (95–100%) and developed to the two-cell stage (89–100%). No differences were found between the physiological media. The majority of oocytes fertilized with spermatozoa frozen in CZB medium developed to blastocysts (80–94%) but development was significantly reduced after fertilization with spermatozoa frozen in PBS and isotonic saline especially in the absence of raffinose (69 and 70% versus 51 and 50%). Normal fertile offspring were obtained in all treatments but there were significantly fewer offspring with spermatozoa stored at −196°C in isotonic saline with or without raffinose and CZB with raffinose. Testicular spermatozoa were extremely sensitive to cryodamage: about 50% frozen to −196°C in CZB with or without raffinose disintegrated after thawing. Almost 100% of oocytes injected with sperm heads from intact (at light microscope level) testicular spermatozoa developed to the two-cell stage but development to blastocysts was reduced significantly compared with that of controls especially those without raffinose. The data indicate that cryopreservation of sperm nuclei requires less stringent conditions than those for the retention of normal physiological function of intact spermatozoa. Motility and plasma membrane integrity are not essential for fertilization and the production of live offspring when nuclei of nonviable spermatozoa are injected into oocytes.

Introduction

The mouse is the primary research animal in mammalian genetics providing models for the analysis of embryonic development and human genetic diseases. Methods to cryopreserve the gametes and embryos are of paramount importance for the maintenance of the large variety of unique inbred, mutant, congenic, recombinant and the rapidly increasing number of transgenic stocks. Storage provides an insurance against the risk of loss through disease as well as economy and ease of transportation.

The advantages of storing spermatozoa include ease of collection, the availability of large numbers of gametes from a single male and the ability to produce new combinations of genetic material by AI or IVF after thawing. However, in contrast to the successful cryopreservation of mouse oocytes (Whittingham, 1977) and preimplantation embryos (Whittingham et al., 1972) attempts to store mouse spermatozoa have met with varying degrees of success (Fuller, 1997).

The first successful cryopreservation of mouse spermatozoa was reported in 1990 (Okuyama et al., 1990; Tada et al., 1990) using raffinose (18%) plus skim milk (3%) or raffinose (18%) plus glycerol (1.75%), respectively, for cryoprotection. To date live offspring have been obtained from cryopreserved mouse spermatozoa from inbred (Tada et al., 1990, 1993; Nakagata and Takeshima, 1993; Penfold and Moore, 1993; Songasen and Leibo, 1996; Songasen et al., 1997), outbred (Okuyama et al., 1990; Tada et al., 1990), hybrid (Yokoyama et al., 1990; Nakagata and Takeshima, 1993; Songasen et al., 1997), transgenic (Nakagata et al., 1992) and wild (Nakagata et al., 1995) mice. Difficulties have been experienced in achieving high levels of fertility with spermatozoa frozen and thawed by the published techniques (Fuller, 1997; Fuller and Whittingham, unpublished observations; Penfold and Moore, 1993; Penfold et al., 1995; Songasen et al., 1997). Although the protocols for freezing differ considerably between the different reports, the
best results were obtained when raffinose and skim milk or glycerol were included in the freezing solution.

In the original study of intracytoplasmic sperm injection (ICSI) into mammalian oocytes, Uehara and Yanagimachi (1976) showed that human spermatozoa frozen in isotonic saline alone decondensed and formed pronuclei (PN) when injected into hamster oocytes indicating that the sperm nucleus is an extremely stable organelle. More recently, the genetic integrity of mouse spermatozoa and their isolated heads was shown to be maintained after freezing and thawing in media containing glycerol by the birth of live young after injection into oocytes (Kuretake et al., 1996). Both whole spermatozoa and sperm heads were considered to be nonviable as the plasma membranes were severely damaged and disrupted. The birth of live offspring has also been reported after freezing spermatozoa in the absence of a cryoprotectant and injection into oocytes after thawing, but apart from being highly successful in the sawfly Athalia rosae (Hatakeyama et al., 1994) only a few births have been achieved in mammals (cattle, Goto et al., 1990; humans, Hoshi et al., 1994, 1995).

Here, we report that mouse spermatozoa frozen with and without a cryoprotectant and considered to be nonviable are highly fertile when the sperm heads are injected microsurgically into oocytes.

**Materials and Methods**

**Reagents**

All inorganic and organic compounds were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise stated.

**Media**

The medium for culturing oocytes after intracytoplasmic sperm injection (ICSI) was CZB medium (Chatot et al., 1989, 1990) supplemented with 5.56 mmol D-glucose l−1 and 5 mg BSA ml−1 (fraction V, Calbiochem, San Diego, CA). The medium for oocyte collection from oviducts and sperm injection was a modified CZB with 20 mmol HEPES l−1, a reduced amount of NaHCO3 (5 mmol l−1), and 0.1 mg polyvinyl alcohol ml−1 (PVA: cold water soluble, Mw 30–50 kDa) instead of BSA. This medium was called HEPES–CZB. The wall of the injection pipette remained less sticky and less mineral oil and cell debris adhered to it when PVA replaced BSA. This was beneficial for the repeated use of a single pipette. CZB and HEPES–CZB media were maintained in 5% CO₂ in air, or air, respectively.

In addition to CZB medium, Dulbecco's phosphate-buffered saline (PBS) and isotonic (0.9%, w/v) NaCl solution were compared for freezing since mouse spermatozoa remain highly fertile after cooling in PBS (Fuller and Whittingham, 1996, 1997) and human spermatozoa frozen and thawed in isotonic saline formed pronuclei after injection into hamster oocytes (Uehara and Yanagimachi, 1976). The final concentration of the cryoprotectant D(+)-raffinose was 18% (w/v) when present in the freezing solutions.

**Preparation of oocytes**

B6D2F1 (C57BL/6 × DBA/2j) hybrid females (6–12 weeks old – from the National Cancer Institute, USA) were injected with 5 IU eCG followed by 5 IU hCG 48 h later. Oocytes were collected from oviducts about 15 h after the injection of hCG. They were freed from the cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (300 USP units mg−1; ICN Biochemicals, Costa Mesa, CA) in HEPES–CZB. The oocytes were rinsed and kept in CZB medium for up to 4 h at 37°C in an atmosphere of 5% CO₂ in air before the sperm injection.

**Freezing epididymal spermatozoa**

For each experimental replicate, one cauda epididymis was removed from a mature B6D2F1 male (8–9 weeks old). The blood and adipose tissue were removed from the surface and the caudal (enlarged) part excised with a pair of fine scissors. This was compressed with forceps to release a dense mass of spermatozoa into a petri dish. Drops (about 2 µL) of spermatozoa were placed in the bottom of 1.5 ml polypropylene centrifuge tubes and overlaid with 0.5 ml warm CZB, PBS or isotonic NaCl. After about 20 min at 37°C the upper 0.2 ml of medium was collected and examined and >90% of spermatozoa were found to be actively motile. The sperm concentration in the medium was 3 × 10⁶ ml−1. An aliquot (100 µL) of the sperm suspension was transferred to a 1.5 ml polypropylene microcentrifuge tube (Fisher Scientific, Pittsburg, PA) and mixed thoroughly with an equal volume of CZB, PBS or isotonic NaCl solution with or without 36% (w/v) D(+)-raffinose (final concentrations of raffinose were 18% and 0% (w/v) respectively). Aliquots (50 µL) of each suspension were dispensed into labelled 1 ml cryogenic vials (A/S NUNC, Copenhagen). Each vial was tightly capped and placed directly into a −20°C or −50°C freezer or liquid nitrogen (−196°C). All samples were stored for periods ranging from 1 day to 4 weeks.

**Freezing testicular spermatozoa**

The seminiferous tubules of the testes were minced in CZB medium to release spermatozoa and other spermatogenic cells. They were resuspended in CZB and prepared for freezing with or without raffinose before immersion in liquid nitrogen for storage.

**Thawing and examination of spermatozoa**

Vials were removed from the freezer or liquid nitrogen and placed in water or air at 24–26°C for about 10 min. A sample of the thawed sperm suspension was examined for motility and viability. The latter was assessed with a commercially available sperm viability test kit (Live/dead FertiLight; Molecular Probes, Inc., Eugene, Oregon) which differentiates between plasma membrane intact and damaged cells according to the fluorescent staining pattern observed with a UV microscope. The nuclei of ‘live’ spermatozoa with intact plasma membranes fluoresce bright green, whereas those of ‘dead’ spermatozoa.
with damaged plasma membranes fluoresce orange-red. About one thousand spermatozoa were examined for each sperm sample.

**Intracytoplasmic sperm injection (ICSI)**

ICSI was carried out with modifications of a technique described by Kimura and Yanagimachi (1995). The thawed sperm suspension (50 μl) was mixed thoroughly with 1 ml of HEPES-CZB medium containing 12% (w/v) polyvinyl pyrrolidone (PVP, M, 360 kDa). A drop of medium containing the thawed spermatozoa was transferred into the micromanipulation chamber on the microscope stage. A single spermatozoon was drawn first into the injection pipette and moved back and forth until the head—midpiece junction (the neck) was at the opening of the injection pipette. The head was separated from the midpiece and tail with application of one or more Piezo pulses (Kimura and Yanagimachi, 1995). After discarding the midpiece and tail the head was redrawn into the pipette and injected into an oocyte. The whole procedure was performed in HEPES—CZB within 1 h of thawing the spermatozoa. The injected oocytes were transferred into CZB medium under mineral oil and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

**In vitro insemination of oocytes**

Cumulus-intact oocytes collected from oviducts of B6D2F1 females about 15 h after the injection of hCG were inseminated with frozen—thawed or fresh epididymal spermatozoa according to the method described by Toyoda et al. (1971). Oocytes were examined for evidence of fertilization 6–8 h after insemination (see below).

**Examination of oocytes and embryos**

Between 5 and 7 h after sperm injection, oocytes were examined with an inverted microscope. Oocytes with two distinct pronuclei and a second polar body (2PN + PB2) were considered to be normally fertilized and were selected for continuous culture for up to 120 h to evaluate embryonic development. Samples of the oocytes were mounted between a slide and coverslip, fixed, and stained for more detailed cytological examination (Yanagida et al., 1991).

**Embryo transfer**

Two-cell embryos developing from the normally fertilized oocytes 24 to 30 h after sperm head injection were selected randomly and transferred to the oviducts of foster mothers on day 1 of pregnancy or pseudopregnancy. Foster mothers were either Swiss Webster (albino) females mated during the previous night with fertile males of the same strain or ICR (albino) females mated with vasectomized males (Kimura and Yanagimachi, 1995). On average about 10 embryos (8–12) were transferred to each recipient female. Pregnant females were allowed to deliver and raise their own pups (red eyes and non-pigmented white coats) as well as foster pups (black eyes and pigmented coats), while the pseudopregnant females only had foster pups with black eyes and pigmented coats to raise. A male and a female pup were selected randomly from each experimental group (see Table 2) and mated when mature.

**Statistical analysis**

Data from the different treatments were compared with Chi squared analysis using Yates’ correction for continuity.

**Results**

All epididymal spermatozoa frozen in CZB, PBS or isotonic saline were non-motile immediately after thawing except for a few (1–2%) with weakly twitching tails in samples frozen rapidly to −196°C in the presence of raffinose. The staining for live and dead spermatozoa revealed that only 2–3% of spermatozoa frozen to −196°C in CZB plus raffinose were ‘alive’. However, they did not fertilize any of 118 oocytes with a conventional in vitro insemination technique (two experiments). Fresh unfrozen cauda epididymal spermatozoa frozen > 90% of oocytes (n = 120) under the same insemination conditions. Epididymal spermatozoa frozen to −50°C in the presence of raffinose were immotile, but staining for live or dead spermatozoa revealed about 3% of spermatozoa were ‘alive’ i.e. with intact membranes. All spermatozoa frozen in the absence of raffinose to −20°C, −50°C and −196°C were diagnosed as ‘dead’ by the sperm staining technique. Fertility was not tested by the conventional IVF technique because all of the spermatozoa were non-motile.

When the heads of frozen—thawed epididymal spermatozoa were injected individually into oocytes, almost all of the oocytes were activated and were fertilized normally (95–100%) and developed to the two-cell stage (89–100%), regardless of the final storage temperature (−20°C, −50°C or −196°C), the presence or absence of raffinose and the type of suspending medium (Table 1). The majority of oocytes fertilized with spermatozoa frozen in CZB medium developed to blastocysts (80–94%) but development was significantly reduced after fertilization with spermatozoa frozen in PBS and isotonic saline especially in the absence of raffinose (69 and 70% versus 51 and 50%). Although blastocyst development was significantly lower (p < 0.05) for spermatozoa that were frozen to −50°C without raffinose when compared with all other treatments with CZB as the suspending medium, it is unlikely to have any biological significance as the responses are all very high and the data are confounded by the difficulties inherent in making simultaneous contemporary comparisons of all treatments. Moreover the subsequent viability after embryo transfer did not reflect the lower development to the blastocyst stage in vitro for spermatozoa stored at −50°C without raffinose.

The proportion of two-cell embryos developing into live offspring after transfer to foster mothers is summarized in Table 2. Normal offspring were obtained in all treatments but there were significantly fewer offspring with spermatozoa stored at −196°C in isotonic saline with or without raffinose and CZB with raffinose, but again the biological significance of these differences is questionable. Approximately equal numbers
of male and female pups were born from all treatments. All the animals were fertile and the females produced litters of normal size with no apparent malformations.

Unlike epididymal spermatozoa, testicular spermatozoa appeared much more sensitive to freezing by the same technique. About 50% of testicular spermatozoa disintegrated after freezing and thawing with or without raffinose. However, almost 100% of the oocytes injected with sperm heads from intact (at the light microscope level) testicular spermatozoa were activated and developed to the two-cell stage (Table 3) but there was a significant reduction in the development of embryos derived from frozen–thawed testicular spermatozoa to the blastocyst stage. This was more marked with spermatozoa frozen in the absence of raffinose. In both treatments most embryos arrested in development between the eight-cell and morula stages.

Discussion

The present study shows clearly that sperm motility and plasma membrane integrity after freezing and thawing are not essential for fertilization and development to live offspring provided the so-called non-viable spermatozoa are injected directly into the oocyte after thawing. Although this is a possible alternative to conventional methods of cryopreservation which attempt to achieve full restoration of sperm function upon thawing, it does little to increase our basic understanding of the cause and type of cellular damage incurred during freezing or thawing. Nevertheless, the present technique provides the first opportunity to conserve spermatozoa from mammalian species when conventional methods of sperm freezing fail to produce motile and fertile spermatozoa upon thawing.
There are several reasons for the apparent high sensitivity of mouse spermatozoa to freezing injury. First, the water permeability of the plasma membrane is reported to be 10- to 50-fold lower than that of other mammalian spermatozoa (Noiles et al., 1995); second, the sperm tail is large in comparison with that of other mammals; third, the total volume and water content (60%) of the mouse spermatozoon (Du et al., 1994) is about twice that of the human (Kleinhaus, et al., 1992) and bovine spermatozoon (Hammerstedt et al., 1978); and fourth, the fragility of the plasma membrane may be increased during the phase transition that has been observed between 4° and 0°C (Noiles et al., 1995).

To date, the highest rates of fertilization in vitro with conventionally cryopreserved spermatozoa have been obtained with certain inbred (DBA/2N - up to 85%, Tada et al., 1990, 1993) and outbred (ICR - up to 95%, Nakagata and Takeshima, 1992) mouse strains. Cryoprotection was afforded by mixtures of 18% (w/v) raffinose and 1.75% glycerol (Tada et al., 1990, 1993) or 18% (w/v) raffinose and 3% (w/v) skim milk (Nakagata and Takeshima, 1992). However, the success of fertilization by frozen-thawed mouse spermatozoa is dependent on the strain and species of mouse (Tada et al., 1990, 1993; Nakagata and Takeshima, 1993; Nakagata et al., 1995), the combination of cryoprotectants (Tada et al., 1990; Penfold and Moore 1993; Songasen et al., 1997), cooling and warming procedures (Tao et al., 1995) and the addition and removal of the cryoprotectants (Nakagata and Takeshima, 1992; Tada et al., 1993). All these variables appear to have contributed to the varying degrees of success that have been achieved in many laboratories attempting to store mouse spermatozoa.

The method of freezing used in the present study was not designed to keep mouse spermatozoa alive. The addition of raffinose, a common cryoprotective component of most solutions used in the development of techniques to freeze mouse spermatozoa, was examined because of its suggested dehydrating and membrane stabilizing ability. In fact, only about 2–3% of spermatozoa were 'alive' by the sperm staining assay after freezing to −50° or −196°C in the presence of raffinose, and 100% of the spermatozoa were judged to be 'dead' in the absence of the cryoprotectant. Although our method of freezing–thawing did not produce fertile spermatozoa as assayed by a conventional in vitro fertilization procedure, we were able to achieve almost 100% normal fertilization (2PN plus PB2) when the heads (nuclei) of the 'dead (killed) spermatozoa were injected into oocytes. As reported by Kuretake et al. (1996), no decrease was found in the ability of sperm heads to activate oocytes after freezing and thawing, indicating that sperm-borne activating proteins, such as oscillin (Parrington et al., 1996), were not lost from the sperm head during one cycle of freezing and thawing. The development of normal fertile offspring from the oocytes indicated that the death of a spermatozoon does not necessarily imply the destruction of the ability of its nucleus to participate in fertilization and embryogenesis. Relatively few live births have been reported after the injection of entire frozen–killed bovine (Goto et al., 1990), human (Hoshi et al., 1994) and mouse (Kuretake et al., 1996) spermatozoa.

According to Da Silva et al. (1992), water contributes as much as one third of the volume of the nucleus of a mature rat spermatozoon. Assuming that the mouse sperm nucleus contains a similar amount of water, we anticipated that freezing mouse spermatozoa without cryoprotectants would damage the sperm DNA. However, apparently this is not the case, at least for sperm heads from spermatozoa frozen in CZB medium to −196°C without any cryoprotection, since their ability to produce normal live offspring was similar to that of sperm heads of unfrozen, intact spermatozoa (Table 2). However, storage of spermatozoa in PBS or isotonic saline significantly reduced blastocyst formation and the proportion of live offspring. This was more marked for blastocyst development in the absence of raffinose, which implies that the cryoprotectant may afford some protection to the sperm DNA. The presence of albumin in CZB medium may also afford some protection to the sperm DNA in the freezing methods used in this study and clearly warrants further investigation. Rybouchkin et al. (1996) reported a tenfold increase in the incidence of chromosomal structural abnormalities in human spermatozoa frozen and thawed twice in a calcium-free medium containing albumin but without cryoprotection. Apart from the trauma to the sperm DNA that might arise from two cycles of freezing and thawing, especially in the absence of calcium, human sperm DNA may be more sensitive than that of the mouse to cryoinjury, since about 15% of nuclear proteins in mature human spermatozoa are histones (Tanphaichitr et al., 1978) compared with less than 2% in mouse spermatozoa (Balhorn et al., 1977). In the DNA–histone complex, up to 40 bp of every 200 bp are not bound to histone (McGhee and Felsenfeld, 1980; Finch and Kug, 1976; Ward, 1993). This portion of the DNA may be

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Table 3. Preimplantation development of mouse oocytes injected with sperm heads from testicular spermatozoa stored at −96°C with and without cryoprotection

<table>
<thead>
<tr>
<th>Final storage temperature (°C)</th>
<th>Freezing medium</th>
<th>Raffinose (18% w/v)</th>
<th>Number of oocytes injected</th>
<th>Number of surviving oocytes (%)</th>
<th>Percentage of surviving oocytes developing in culture</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Oocytes with 2PN + PB2 at 8 h</td>
</tr>
<tr>
<td>Fresh (control)</td>
<td>CZB</td>
<td></td>
<td>83 (4)</td>
<td>72 (87)</td>
<td>99</td>
</tr>
<tr>
<td>− 196</td>
<td>CZB</td>
<td>+</td>
<td>76 (4)</td>
<td>66 (87)</td>
<td>98</td>
</tr>
<tr>
<td>− 196</td>
<td>CZB</td>
<td>−</td>
<td>79 (4)</td>
<td>74 (94)</td>
<td>97</td>
</tr>
</tbody>
</table>

1Time (h) after sperm head injection. Significant χ² comparisons with control. aP < 0.025, bP < 0.001, and between presence and absence of cryoprotectant cP < 0.001.
PN: pronuclei; PB2: second polar body.
more susceptible to various physical and chemical stresses than the protamine-bound DNA. Therefore, human spermatozoa, which contain a higher concentration of histones than do mouse spermatozoa, might be more vulnerable to physical stresses such as freezing and thawing. This contention is further supported by our previous studies which have shown that mouse sperm nuclei are less susceptible to sonication (Kuretake et al., 1996) than are human spermatozoa (Martin et al., 1988).

It is interesting to note that the nuclei of mouse testicular spermatozoa, unlike those of mature epididymal spermatozoa, are more vulnerable to freezing with and without cryoprotectant. The extensive -SS- crosslinking of sperm nuclear protamines during epididymal maturation (Bedford and Calvin, 1974) apparently makes sperm nuclei resistant to freezing and thawing injury. The testicular spermatozoa that were intact after freezing and thawing activated and fertilized oocytes in high proportions similar to those of epididymal spermatozoa (overall 98% (137/140) versus 99% (477/482)), but the developmental capacity of the resulting embryos was significantly reduced. The greatest reduction in blastocyst formation occurred with embryos derived from the injection of sperm heads from testicular spermatozoa frozen in the absence of raffinose. Development was arrested between the eight-cell and morula stages, suggesting possible injury to the testicular sperm DNA during freezing or thawing, which results in disruption of the new embryonic genome activity essential for normal preimplantation development. The partial improvement in development after cryopreserving the testicular spermatozoa with raffinose suggests that some dehydration of the sperm nucleus produced by exposure of spermatozoa to the cryoprotectant before freezing might help avoid injury due to excess ice formation during freezing and thawing.

The concentration of actively motile spermatozoa has to be high ($>10^4$ or $10^5$ ml$^{-1}$) for normal fertilization to take place in vitro. In contrast, ICSI requires only one spermatozoon per oocyte to effect fertilization and it is not necessary for the spermatozoa to be motile or alive as long as the nuclei are 'intact' in terms of reproductive and genetic potential. Moreover, the methods for freezing whole spermatozoa simply to preserve the integrity of the sperm nucleus are much less stringent than those required to produce viable motile spermatozoa for insemination and IVF. Recently we obtained live offspring from spermatozoa frozen to $-196^\circ$C in the intact cauda epididymis. Three male mice were killed and kept at $0^\circ$C for 1–3 days. The cauda epididymides were removed from the mice, placed in 1.5 ml polypropylene vials without suspending medium and plunged into liquid nitrogen. Three days later, the epididymides were thawed at room temperature ($22^\circ$C) and the spermatozoa released into CZB medium. All spermatozoa were 'dead' when examined after staining. Three normal offspring were obtained after the injection of oocytes with sperm heads from the 'dead' spermatozoa indicating that, although less efficient than fresh sperm preparations, this technique may be an important means of conserving rare and unique genetic material when an animal dies suddenly or is inadvertently killed (unpublished observations).

In conclusion, the simple radical method of freezing mouse spermatozoa and the injection of sperm heads after thawing has wide implications as an alternative means of storing male gametes in the animal kingdom. This is especially true for mammals when the number of spermatozoa available for insemination may be limited or the spermatozoa refractory to cryopreservation, for example newly created transgenic and mutant animals, rare and endangered breeds and species and in certain forms of human male infertility.

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