MARINE BIOTECHNOLOGY

Intracytoplasmic Sperm Injection Using Cryopreserved, Fixed, and Freeze-Dried Sperm in Eggs of Nile Tilapia

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Abstract

Gamete preservation techniques are essential in animal husbandry as well as in assisted reproduction for humans. In this research we attempted to use 3 different sperm preservation techniques in combination with newly developed techniques for intracytoplasmic sperm injection (ICSI) to fertilize eggs of a teleost fish, the Nile tilapia (*Oreochromis niloticus*). Of 47 eggs injected with fresh sperm, 11 (23%) were fertilized, 5 developed abnormally, and 4 developed normally and hatched; from these, one grew to adulthood. Nuclear DNA content of 4 of the abnormal embryos indicated that they were diploid. Flow cytometric analysis of a blood sample from the surviving ICSI fish collected 2 months after fertilization indicated that the fish was diploid. Of 45 eggs injected with cryopreserved sperm, 9 (20%) developed to the blastula stage. Of 40 eggs injected with sperm preserved in 70% methanol, none were fertilized. No injections were possible with freeze-dried Nile tilapia sperm owing to technical difficulties during manipulation. Although the findings described here are limited, they provide the first steps toward using sperm preservation methods in addition to cryopreservation for fertilization in fishes.

Key words: ICSI — Nile tilapia — fertilization — sperm — eggs — cryopreservation

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Introduction

Sperm storage began to be applied commercially in livestock shortly after the first calf produced from cryopreserved semen was born in 1951 (reviewed by Curry, 2000). Currently, industries such as dairy are based on the use of artificial insemination and frozen sperm. Although artificial insemination with cryopreserved sperm provides advantages over natural fertilization, not all farm animal industries have incorporated this assisted reproductive technology. In aquaculture, for example, cryopreservation has been studied in an estimated 200 species of fish (Rana, 1995), but it has not become established in research or commercial applications (Tiersch, 2000). This will likely change in the near future given that aquaculture is the fastest growing sector of agriculture and overfishing and human development are threatening dozens of species of fish (Hutchings, 2000; Pauly et al., 2002). The establishment of repositories for genetic material is seen as a way of maintaining biological diversity, developing broodstocks, and reducing space used in hatcheries for maintenance of males (Holt, 1997; Tiersch, 2000; Cloud et al., 2000).

At present cryopreservation is the only reliable way to preserve fish sperm indefinitely; however, it is expensive and potentially unreliable because it requires a constant supply of liquid nitrogen. A good alternative would be the use of freeze-dried sperm or sperm preserved in alcohol, each of which could be stored at 4°C or at room temperature (25°C). The concept of using freeze-drying as a sperm storage technique was first introduced 50 years ago with reports of unsuccessful attempts to obtain viable avian sperm after vitrification and dehydration (Polge et al., 1949). It was not until 1957 that the first live births of rabbits were reported from freeze-dried

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spermatozoa (reviewed by Larson and Graham, 1976). However, attempts in other laboratories were not successful (Saacke and Almquist, 1961; Larson and Graham, 1976). At that time the aim of preservation was to maintain the integrity or viability of sperm cells for later use in in vitro fertilization. However, after the physical and chemical treatments, sperm motility was compromised, limiting the use of this technique.

The introduction of sperm microinjection techniques in mammalian species (Uehara and Yanagimachi, 1976, 1977) revitalized the notion of noncryogenie sperm preservation. It was shown that freeze-dried sperm injected in mice oocytes could produce normal offspring (Wakayama and Yanagimachi, 1998; Kusakabe et al., 2001). Others reported that injection of sperm chemically dehydrated in 100% ethanol, 100% methanol, chloroform-methanol, and Carnoy's fluid were capable of developing into male pronuclei (Katayose et al., 1992; Tateno et al., 1998). After these treatments sperm are considered to be conventionally ''dead'' but possess genetically normal nuclei, capable of supporting development. Although these treatments destroyed the cell membrane, the sperm did not need to be motile if intracytoplasmic sperm injection (ICSI) was used to produce fertilization.

The goal of the present study was to evaluate the possible use of sperm preservation methods in combination with ICSI for use with Nile tilapia (*Oreochromis niloticus*), an important aquaculture species throughout the world. The objectives of this experiment were to evaluate ICSI with (1) cryopreserved sperm, (2) sperm fixed in a 70% solution of methanol, and (3) freeze-dried sperm.

Materials and Methods

Animals. Nile tilapia females were held individually in 80-L tanks in a ''single-breeding'' system at 26°C (Poleo, 2002). Salinity was maintained at 1.5 mg/L, and alkalinity and hardness at approximately 200 mg/L as $CaCO_3$. Females that showed a swollen abdomen and projecting genital papilla were selected for strip spawning. Fish were anesthetized with tricaine methanesulfonate (methyl-*m*-aminobenzoate, MS222) (Argent Laboratories) before being stripped of eggs. The abdominal region of the fish was dried with paper towels and squeezed gently, starting behind the pectoral fins and moving toward the tail. Ripened eggs were stripped easily and showed uniformity of size. The presence of blood, connective tissue, or size variation indicated that eggs were not ripe, and they were not used for these experiments.

Sperm were collected by gently squeezing the sides of unanesthetized males. A 10-μL pipette tip connected to a mouth pipette was used to extract sperm, which were diluted 1:1 in Hanks' balanced salt solution (HBSS) (280 mOsmol/kg) in 1.5-ml microcentrifuge tubes and placed on ice. Motility was assessed by placing 10 μl of sperm dilution on a glass microscope slide and mixing with 20 μL of distilled water. The sperm mixture was observed at 200× by use of dark-field microscopy (Optiphot 2, Nikon). Sperm that actively moved forward were considered to be motile. Only samples of sperm showing 90% or greater motility were used in these studies.

Sperm Injections. Injections were performed using a micromanipulator apparatus consisting of an inverted microscope (Diaphot Nikon) equipped with 2 mechanical micromanipulator units (Leitz), which moved either the holding or injection pipettes. Each pipette was connected to a screw syringe by fine-bore Teflon tubing filled with light mineral oil (Sigma Chemical Corp.). The injection and holding pipettes were made from borosilicate glass capillary tubes (Sutter Instrument Company). The injection pipette had an internal diameter of approximately 15 μm. Owing to variability in size among egg (1.5–2.5 mm) batches collected from different females, the holding pipettes were prepared for each batch of eggs with internal diameters ranging from 200 to 400 μm. When eggs were correctly positioned with the animal pole facing outward, the injection pipette was pushed through the micropyle (a single natural opening for sperm entry into the egg) into the cytoplasm and a sperm cell was injected with a small volume (aprox. 7 pl) of embryo medium buffer (EMB: 13.7 mM NaCl, 5.40 mM KCL, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.30 mM $CaCl_2$, 1.00 mM $MgSO_4$, 4.20 mM $NaHCO_3$) (Westerfield, 1995) containing 5% polyvinylpyrrolidone (PVP), 360,000 molecular weight (Sigma Chemical Corp.), to prevent sperm from sticking to the wall of the pipette. Sperm cells were always injected nonmotile after being activated by placing 2 μl of sperm suspension in 2 μl of EMB containing 5% PVP. As a control treatment, eggs were injected with approximately 7 pl of EMB containing 5% PVP. In addition, egg quality was evaluated by artificial insemination of approximately 50 eggs with fresh sperm. Eggs from batches with 70% or greater fertilization with fresh sperm were included in the analysis. Eggs were evaluated 4 hours after injection, and those that presented well-developed blastomeres (8-cell, stage 4) (Galman and Avtalion, 1989) were counted as fertilized.

DNA Content Analysis. DNA content was analyzed using a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled 480-nm argon laser. The FACSComp software (Becton Dickinson) was used to calibrate the instrument settings. The yolk sacs of 4-day-old embryos were removed, and the remaining tissue was disrupted by passage 3 times through a 25-gauge syringe needle after suspension in 0.5 ml of lysis buffer containing 25 μg of buffered RNase, 0.1% sodium citrate, and 0.1% Triton X-100 (Tiersch and Chandler, 1989). The solution was filtered through 20-μm nylon mesh. Blood was collected from the caudal vein of a fish 3 months after production by ICSI. For analysis an aliquot of 250 μl of disrupted embryo or 1 μl of blood was diluted in lysis-staining buffer, which included 25 μg of propidium iodide. Blood cells from channel catfish *Ictalurus punctatus* (1.98 pg of DNA per cell) (Tiersch et al., 1990) and zebrafish *Danio rerio* (3.15 pg of DNA per cell) (Poleo et al., 2001) were used as internal references. Measurements of the DNA content of normal diploid Nile tilapia (2.10 ± 0.09 pg of DNA per cell) (Poleo, 2002) were compared with those of larvae produced by ICSI.

Cryopreservation of Sperm. Sperm were cryopreserved by following the procedure of Rana and McAndrew (1989) with modifications as described below. Briefly, sperm were diluted 1:10 in HBSS containing a final concentration of 10% methanol and stored in 0.5-ml straws. A 10-ml syringe coupled to rubber tubing was used to fill the straws. The open ends of the straws were sealed with polyvinyl chloride powder and cooled to −80°C in a controlled-rate freezer (Kryo 10 series, Planer products) at a rate of 40°C/min. The straws were plunged into liquid nitrogen for storage. After a week the contents of single straws were used for ICSI. Straws were thawed in a 40°C water bath for 8 seconds. Both ends of the straws were cut, and the sperm solution was expelled into a 1.5-ml microcentrifuge tube and held on ice. For injections, within 5 minutes of thawing an aliquot was diluted 1:20 in HBSS to reduce the methanol concentration to 0.5%. Washing by centrifugation was not done to avoid further damage to the sperm. Artificial insemination was conducted using cryopreserved sperm with eggs of Nile tilapia. To observe the possible effect of the low concentration of methanol (0.5%), 20 eggs were artificially inseminated and, after 5 minutes, injected with buffer containing 0.5% methanol and 5% PVP.

Methanol Fixation of Sperm. Sperm were collected as described previously. The sperm solutions were diluted in methanol to a 70% final concentration and kept at 4°C for 24 hours. Before injection an aliquot of the 70% solution was diluted 1:200 in HBSS to reduce the methanol concentration to 0.35%. A group of 50 eggs from every batch was artificially inseminated using treated sperm. Only eggs that came from batches with greater than 70% fertilization were included in the analysis.

Sperm Freeze-drying Treatment. Sperm were collected and cryopreserved as described above. The straws were removed from liquid nitrogen, clipped in half, and immediately placed in a precooled (−80°C) 800-ml flask (Labconco Corp.), which was connected to a lyophilizer (Lyph-Lock 18, Freezedry/Shell Freeze System, model 77555-01, Labconco). Samples were dried overnight and stored in a sealed container at 4°C. Samples were prepared for injection by rehydrating them with 500 μl of distilled water and diluting 1:1 with EMB containing 10% PVP. Artificial insemination was performed for every session of injections by incubating rehydrated sperm with 50 Nile tilapia eggs.

DNA Staining. Freeze-dried sperm were hydrated with water containing 5 μg/ml of Hoechst 33342 (Sigma). Ten-microliter drops were placed on a glass microscope slide and observed at 400× magnification under ultraviolet light (Nikon, Microphot-SA). A sample of fresh sperm was used to compare the size and appearance of the nuclei. The diameters of the sperm were calculated using image analysis software (Optimas 5.1a, Bioscan).

Statistical Analysis. Statistical analysis was performed using SAS software for Windows (Version 8.01, SAS Institute). Differences in the incidence of fertilization among treatments involving ICSI were evaluated using logistic regression with the GENMOD procedure. Logistic regression was modeled as a class of generalized linear models in which the response probability distribution function was binomial. Differences were accepted as significant when P was less than 0.05.

Results

Fresh Sperm. Of the 47 eggs injected with fresh sperm, 11 (23%) were fertilized (8-cell, stage 4) (Galman and Avtalion, 1989), 7 (15%) developed to blastula (stage 8), 5 (11%) developed abnormally to neurula (stage 11), and 4 (8%) developed normally and hatched (Table 1). Two of the hatched embryos developed to active feeding. One of these fish (named ''Sebastian'') remains alive 3 years later at the time this report was written (Figure 1). DNA content of 4

Table 1. Effect of Cryopreservation and Methanol Fixation on Ability of Nile Tilapia Sperm to Fertilize After Intracytoplasmic Sperm Injection[a]

Sperm treatment	*Number of Injections*	*Percentage fertilized*	*Blastula*	*Gastrula*	*Advanced neurula*[b]		*Sacfry*[c]	*Fry*[d]
					Abnormal	*Normal*		
Fresh	47	23	11	9	5	4	4	2
Control	45	0	0	0	0	0	0	0
Cryopreserved	45	20	9	0	0	0	0	0
Control	41	0	0	0	0	0	0	0
Methanol-fixed	40	0	0	0	0	0	0	0
Control	45	0	0	0	0	0	0	0

[a]Control injection were made without sperm. Only eggs that came from batches with a fertilization percentage higher than 70% were included in the analysis.
[b]Embryos that showed a complete neural tube, somites, and optic vesicle (stage 11) (Galman and Avtalion, 1989).
[c]Hatched embryo that did not absorb the yolk sac.
[d]A free-swimming fish with active feeding.

of the abnormal embryos indicated that they were diploid. Flow cytometric analysis of a blood sample from the surviving ICSI fish taken 2 months after fertilization indicated that it was diploid. The normal fish produced from ICSI was a female.

Cryopreserved Sperm. The sperm presented 95% motility before freezing, compared with 60% after thawing. There was no significant difference ($P = 0.6000$) between fertilization using fresh sperm (90% ± 2%) and cryopreserved sperm (89% ± 1%). Of the 45 eggs injected with cryopreserved sperm, 9 (20%) were fertilized (stage 4), and 9 developed to late morula (stage 7) but did not develop beyond this stage (Table 1, Figure 2). None of the 41 eggs injected without sperm showed signs of embryonic development (Table 1).

Methanol-Preserved Sperm. Of the 40 eggs injected with sperm treated with 70% methanol, none were fertilized (Table 1). Twenty eggs inseminated with fresh sperm before injection of HBSS solution containing 0.5% methanol developed normally.

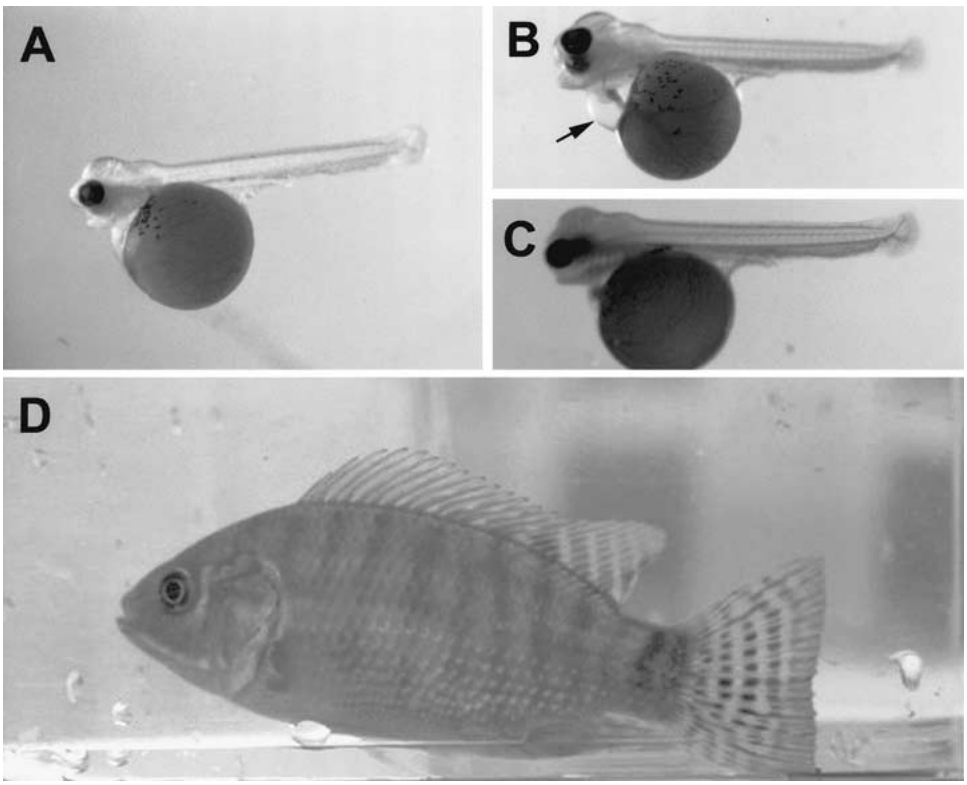

Fig. 1. Intracytoplasmic sperm injection using fresh Nile tilapia sperm. **A:** Nile tilapia sacfry produced from artificial fertilization (noninjected), 3 days after fertilization. **B:** Nile tilapia sacfry produced by ICSI, 3 days after fertilization. The pericardial cavity (arrow) was larger than normal, and the fish died 7 days after hatching. **C:** Nile tilapia sacfry produced by ICSI, 3 days after fertilization, showing characteristics of normal sacfry. **D:** The same fish (shown in C and named ''Sebastian'') 3 months later.

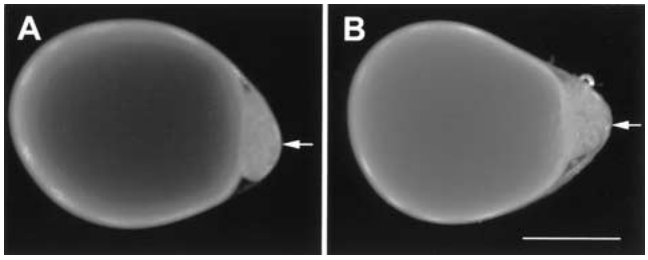

Fig. 2. Intracytoplasmic sperm injection using cryopreserved Nile tilapia sperm. **A:** Morula stage, 6 hours after artificial fertilization (noninjected). **B:** Morula stage, 6 hours after ICSI. The embryos injected with thawed sperm ceased development at this stage. The cells are at the animal pole (arrows) (scale bar, 750 μm).

Freeze-dried Sperm. Injections with freeze-dried sperm were not possible because of the difficulty of visualizing the sperm in the injection buffer. Sperm that were freeze-dried had no tails, and the heads were difficult to localize by light microscopy. Staining of the sperm DNA with Hoechst 33342 showed that the nuclei retained a round morphology and normal size (2.3 ± 0.2 μm) when compared with fresh sperm (2.3 ± 0.2 μm). However, the freeze-dried nuclei had a diffuse appearance (Figure 3).

Discussion

The aim of this work was to evaluate Nile tilapia sperm that were cryopreserved, preserved in methanol, or freeze-dried for use in ICSI. The percentage of ICSI embryos that reached neurula stage (19%) in this study was almost 4 times greater for Nile tilapia than in previous attempts (5%) (Poleo, 2002), possibly because of the additional experience gained in manipulation of the gametes. The phenotype expressed in this study by the adult fish obtained by ICSI (female with wild-type pigmentation) could have resulted from fertilization by the injected sperm or from induction of gynogenesis (all-maternal inheritance) by the injection. Although gynogenesis is possible, the probability of it occurring was low. It has been reported that the production of mitotic gynogenetic *Oreochromis aureas* yielded a mean survival rate of 0.4% ± 0.3% for fish that developed beyond the yolk sac stage, and of these 50% showed abnormal development (Don and Avtalion, 1988). In other reports of Nile tilapia used in gynogenetic experiments, none of the fish developed beyond sacfry (Peruzzi et al., 1993) when fertilized with irradiated *O. niloticus* sperm.

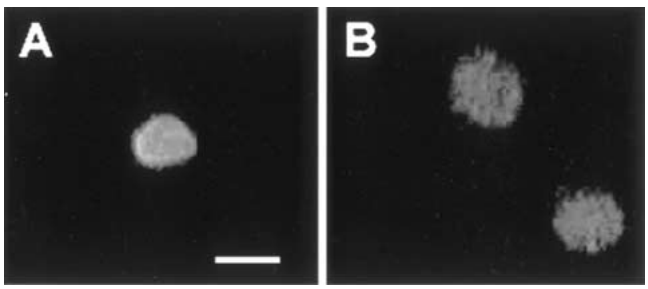

Fig. 3. Fresh (**A**) and freeze-dried (**B**) Nile tilapia sperm stained with Hoechst 33342. Note the diffuse appearance of the freeze-dried nuclei (scale bar, 2.3 μm).

The low percentage of viability and the high percentage of abnormalities (disjunction of the jaws from the body and head enlargement) found in gynogenetic fish have often been attributed to the expression of defective recessive alleles and increased inbreeding (Purdom, 1969; Onozato, 1984; Suzuki et al., 1985; Don and Avtalion, 1988). In this study 9% of the injected eggs developed beyond sacfry without showing major malformations, suggesting that the fish were the result of ICSI. Further evidence could be gathered by comparing specific genetic markers in the genomes of parents and offspring by techniques such as DNA fingerprinting (Carter et al., 1991; Naish et al., 1995; Jenneckens et al., 1999) and random amplification of polymorphic DNA (RAPD) (Van Eenennaam et al., 1996; Jenneckens et al., 1999).

Development to late morula (stage 7) of eggs injected with fresh sperm (23%) or cryopreserved sperm (20%) occurred within 10 hours of injection. All the eggs fertilized using cryopreserved sperm stopped development at blastula. This most likely does not reflect parthenogenetic activation because none of the 82 control eggs injected without sperm developed beyond blastodic formation. Nuclear DNA content analysis was not possible because of the small number of embryos obtained (9) and the limited number of cells present at blastulation.

It is possible that the sperm selected for injections were damaged, compromising further development. Although the effectiveness of cryopreservation has been established for artificial insemination, a percentage of the cryopreserved cells usually suffer some type of injury, as indicated by decreased motility after freezing. However, lack of motility does not necessarily define the fertilization capability of sperm, as demonstrated when eggs injected with nonmotile sperm produced normal fish (Poleo et al., 2001). Nevertheless, ice crystal formation can disrupt the sperm membrane, resulting in loss of cytoplasmic contents that may be necessary for egg activation. Ice crystals can also damage the chromatin, resulting in fragmented DNA. Injection of eggs with sperm containing fragmented chromatin has resulted in failure of sperm decondensation, fertil-

ization, and embryonic development in humans (Sakkas et al., 1996; Dumoulin et al., 2001). There is also the possibility that embryonic development was affected by the methanol used as cryoprotectant (0.5% of the injection volume) that was injected along with the sperm. However, control injections of 0.5% methanol in eggs previously inseminated with fresh sperm did not compromise development.

Injection of sperm preserved in 70% methanol did not fertilize any of 45 Nile tilapia eggs, suggesting that the sperm were not genetically viable. It is known that high concentrations of methanol or ethanol (>70%) do not alter the primary structure of DNA (Sambrook et al., 1989) or the protamine molecules involved in the stability and packaging of the sperm nuclei in hamsters (Lee et al., 1991). However, methanol could affect the interaction between protamines and DNA, or affect other factors involved in activation of the eggs. Human sperm and hamster sperm stored in methanol, ethanol, and chloroform-methanol were capable of decondensation and development into pronuclei after injection into hamster oocytes (Katayose et al., 1992), although this does not ensure the genetic integrity of the sperm nuclei and future embryonic development.

Other experiments in which mouse oocytes were injected with sperm stored in ethanol showed that storage for more than 1 day changed the structure of the sperm chromosomes, compromising the development of the embryo (Tateno et al., 1998). These results suggest that ethanol might have subtle effects on DNA-protamine interactions, inhibiting proper DNA decondensation. This had been further observed by experiments in which mouse epididymal spermatozoa were damaged by freeze-drying if first treated with dithiothreitol (DTT), which reduces S-S bonds (Kaneko et al., 2003). In mammals, sperm nuclei are stabilized by extensive disulfide bonding between the DNA and protamines. In fish, protamines are also present in sperm nuclei and are thought to stabilize the nuclei (Dixon and Smith, 1968; Shimizu et al., 2000). However, there are few disulfide bonds (Yanagida et al., 1991), which could make fish sperm less resistant to alcohol treatment.

Alcohols could also affect factors involved in egg activation and embryonic development. Mouse oocytes do not usually require artificial activation after ICSI. However, oocytes injected with sperm fixed in ethanol required artificial activation to stimulate further development (Kimura et al., 1998; Tateno et al., 1998). It might be useful to artificially activate Nile tilapia eggs after injection with sperm preserved in methanol to see if the same response is observed. This must await development of procedures for artificial activation.

The production of mice by injection of freeze-dried sperm has tested the conventional definitions of ''live'' and ''dead'' sperm (Wakayama and Yanagimachi, 1998; Kusakabe et al., 2001; Ward et al., 2003). Although mouse sperm are considered to be dead when freeze-dried, their fertilization potential is not eliminated, and the sperm factors involved in oocyte activation remain intact. This was shown by the fertilization of mouse oocytes by ICSI without artificial stimulus (Wakayama and Yanagimachi, 1998). No injections of freeze-dried sperm were possible in the present study owing to technical problems related to manipulation of the rehydrated sperm cells. However, the information gathered could serve as the starting point for future experiments. With the protocol used here, the freeze-dried sperm were not motile and not able to fertilize Nile tilapia eggs, most likely because of loss of the tail. Although the stained sperm heads seemed to be the same size as those of fresh sperm, the nuclei appeared more diffuse. Freezing-drying altered the refractive properties of the sperm, preventing visualization with conventional light microscopy. To perform injections using freeze-dried sperm from Nile tilapia, a microscope with phase-contrast filters would be desirable. The use of dyes to stain the sperm or the buffer solution to gain more contrast could also improve manipulation.

In summary, although these results are limited, those obtained with cryopreserved sperm suggest that with a more refined technique, ICSI could yield normal fish from preserved sperm. This could be the first step toward the development of new technologies that would reduce maintenance and the cost of genetic repositories in the future. More basic information on the requirements for fertilization is needed, however, to understand and develop new sperm preservation techniques for fish.

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