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Effects of Cryoprotectants and Freezing Rates on Cryopreservation of Catfish, *Silurus asotus* Sperm

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Milt of the catfish was stripped into immobilizing solution containing 175 mM NaCl and 30 mM Tris at pH 7.8 and was successfully cryopreserved after a stepwise freezing procedure. After stepwise thawing, motility of spermatozoa was slightly lower than that of fresh sperm. Batches of 40-80 eggs were fertilized with cryopreserved spermatozoa, after thawing and activation in solution containing 50 mM NaCl, 20 mM Tris and HCl at pH 7.8; this resulted in 62.2% fertilization success, compared to 70.6 % with fresh sperm.

Keywords: Catfish sperm, Cryopreservation, Cryoprotectant, Stepwise freezing

Introduction

As fish farming expands and harvesting of wild stocks becomes more intense, there is a growing need for techniques of sperm storage to facilitate artificial breeding procedures and to preserve desirable gene pools. The catfish, *Silurus asotus* is an important species in Korea. The development of cryopreservation techniques for other fish sperm has resulted in fertilization success approaching in some cases those obtained with fresh sperm (Scott and Baynes, 1980). Most experimental work in this field have focused on finding optimal diluents, cryoprotectants, thawing solutions, rates of freezing and thawing for carp, *Cyprinus carpio* (Cognie et al., 1989) and salmonids (Rana, 1995; Fabbrocini et al., 2000). Methods have also been developed for cryopreservation of other freshwater fish sperm: tilapia, *Oreochromis mossambicus* (Harvey, 1983), European catfish, *Silurus glanis* (Marian and Krasznai, 1987; Linhart, et al., 1993) and African catfish, *Clarias gariepinus* (Steyn and Van Vuren, 1987). This communication reports on the optimum cryoprotectant and appropriate freezing rate for sperm of the catfish, *Silurus asotus*.

Material and Methods

Broodstock and sperm collection

The catfish were reared in ponds adjacent to Naju City, Chonnam Province. During the pre-spawning period, the parental broodfish were kept separately in small ponds. To collect the milt, the males were anesthetized in lidocain (200 ppm). Accumulated urine and feces were removed. The milt was then obtained by abdominal pressure and stored in sealed test tubes with crushed ice until use.

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Sperm motility

Motility of the sperm from each male was determined immediately after collection. A glass slide was coated with 1% bovine serum albumin and then dried to prevent sperm from sticking to the glass. Using the two-step dilution procedure of Cosson et al. (1989) to synchronous induction of sperm motility, the sperm motility was measured.

The semen was diluted 10-fold in immobilizing solution (175 mM NaCl, 30 mM Tris, pH 7) which also served as an extender for cryopreservation. The diluted sperm was stored in polyethylene tubes on ice during experiments. Motility was estimated (after 1/100 dilution; final dilution 1/1000 considering the first dilution step in the immobilizing solution) by a numerical index (Table 1) of Strûssmann et al. (1994). Fresh milt containing highly motile sperm was pooled and used in the experiments.

Optimum cryoprotectant

One of the following cryoprotectants, glycerol and dimethylsulfoxide (DMSO), was added to the immobilizing solution to formulate the extenders at concentrations ranging from 5 to 45% for freezing. The milt was diluted in the extenders at a ratio of 1:6. The diluted milt was inserted into 0.5 ml plas-

Table 1. Numerical index for the evalution of sperm motility

Index	Motility characteristics
5	Most spermatozoa display rapid movement; impossible to track the course of any spermatozoa
4	Most spermatozoa move rapidly while some move slowly
3	Three classes of spermatozoa can be recognised in equivalent numbers: spermatozoa moving rapidly, spermatozoa moving slowly or vibrating, and those immotile
2	Most spermatozoa are vibrating or immotile, while some present lateral vibration
1	Most spermatozoa are immotile and some present lateral vibration
0	All spermatozoa are immotile

tic straws and frozen at a freezing rate of 40°C/min to -80°C after equilibration for 10 min at room temperature, and subsequently plunged into liquid nitrogen (-196°C). For thawing, the straws were put into a 30 water bath for 15 sec. The motility of frozen-thawed sperm was evlauted in the activation solution (200 μl the activation solution + 2 μl thawed sperm). Motility tests on spermatozoa were carried out in triplicate.

To evaluate the effect of different freezing rates, 15% glycerol was used as a cryoprotectant. Four freezing gradients were tested: (a) 40°C/min to -80°C; (b) 30°C/min to -80°C; (c) 20°C/min to -80°C; (d) 10°C/min to -80°C.

Fertilization success

The milt, diluted in the immobilizing solution, was mixed with 15%, 25% or 35% glycerol, frozen at the freezing rate of 30°C/min to -80°C, and stored at -196°C for 1 h. The fertilizing capacity was tested with eggs taken from three females with three replicates for each of them. Artificial insemination with fresh (diluted) or frozen sperm was carried out with eggs obtained from three different females, stripped at 16 or 20 h after injection of 3 mg carp pituitary extract (CPE) per kg of fish. Batches of 40-80 eggs were placed in 5 cm diameter petri-dishes; the diluted fresh or thawed (500 μl) milt was dropped on the eggs and immediately covered with 5 ml activation solution. Sperm and eggs were left in contact for 5 min. Thereafter, the diluent was discarded, and substituted with fresh water. Eggs in the petri-dish were incubated in hatchery water at 23-25°C. All the water in the petri-dishes was renewed every 6 h.

The success of fertilization was measured as percentage of eggs at the morula stage (formation of 8-64 blastomeres was assessed microscopically). The percentage of developed morulae is a good estimate of the percentage of fertilization when it is carried out with normal sperm.

Data analysis

Statistical analyses were made by the one-way analysis of variance (ANOVA) and Tukey test (Zar, 1984).

Results

Motility of the post-thawed sperm preserved at all the tested DMSO concentrations was too low; hence, it is not a suitable cryoprotectant for the catfish sperm. Conversely, the motility of the frozen spermatozoa at all the tested glycerol concentrations was fairly high (Fig. 1). The highest motility was observed with 15% glycerol, although it was not significantly different from others ($P>0.05$).

Hence glycerol (15%) was selected for freezing trials. Good result (motility index : 3.75) was obtained, when freezing rate of 30°C/min to -80°C was used, although it was not

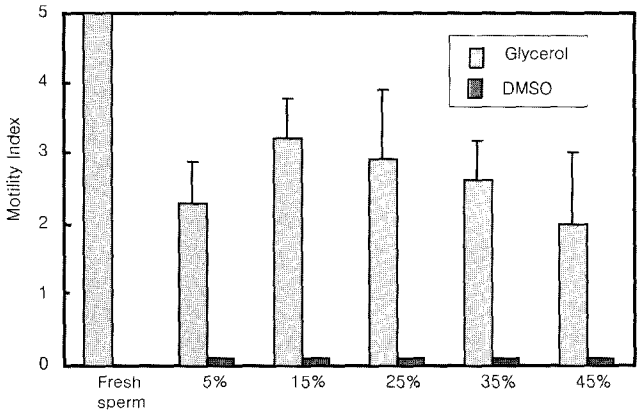

Fig. 1. Motility of catfish sperm after cryopreservation using glycerol and DMSO as cryoprotectants at the concentration ranging from 5 to 45%.

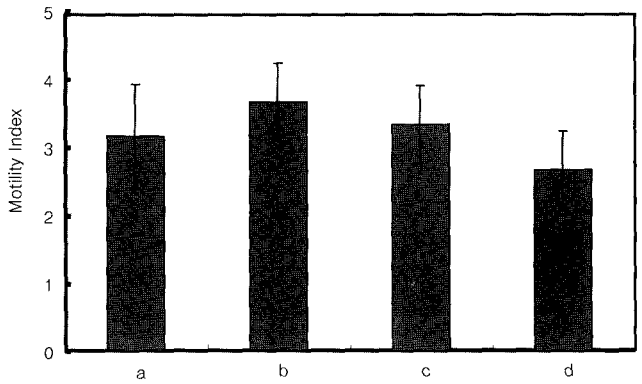

Fig. 2. Motility of catfish sperm after cryopreservation at different freezing rates using 15% glycerol as a cryoprotectant (a: 40°C/min to -80°C, b: 30°C/min to -80°C, c; 20°C/min to -80°C, d; 10°C/min to -80°C).

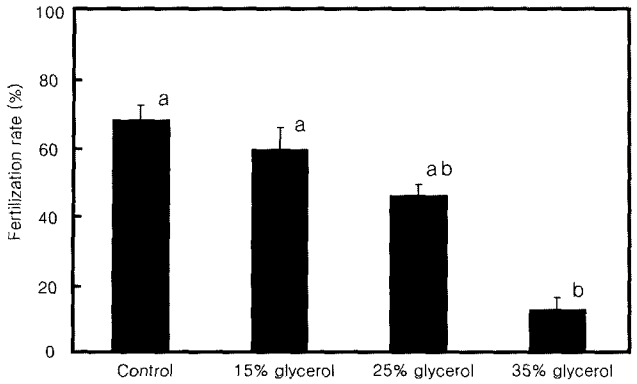

Fig. 3. Effect of 15%, 25% and 35% glycerol on fertilization success of frozen sperm. Different letters on the bars indicate significant-difference ($P<0.05$).

significantly different from others ($P>0.05$) (Fig. 2).

Fertilization success of sperm frozen with 15% glycerol was almost equal to that of diluted fresh sperm (control). The level of success was higher, when frozen with 15% glycerol ($p<0.05$), than at 25 or 35% glycerol (Fig. 3).

Discussion

Since Blaxter (1953) reported on the use of diluted seawater as a diluent for herring , *Clupea pallasi* sperm. Several media were tested as diluents for fish sperm preservation (Kubota, 1961; Hara et al., 1982; Palmer, 1994). In the present study, spermatozoa of the catfish stripped directly in an immobilizing solution was successfully cryopreserved.

Previous observations by Marian and Kraszani (1987) on the European catfish, *Silurus glanis* showed that the fer tilizing capacity of frozen sperm was nearly as good as fresh sperm, but they used a 1,000 fold higher spermatozoa/egg ratio, and the milt was drawn from testis and had not been exposed to the immobilizing solution. Besides, they used larger volume of 100 to 2,000 μl pellets and a lower freezing rate (10°C per min). Their freezing solution was more complex, as it included sucrose or glucose, an advantageous for cryoprotectant for rainbow trout, *Oncorhynchus mykiss* sperm (Holtz et al., 1991). However, our unpublished data on the catfish showed that sucrose or glucose did not improve the sperm motility after thawing. Our observations confirm the previous finding of Padhi and Mandal (1995), who suggested that 10% glycerol could be effective for freezing the milt of Asian catfishes, *Heteropneustes fossilis* and *C. batrachus*.

When cells are subjected to subzero temperatures, they initially supercool while ice is formed in the external medium. The manner, in which cells regain equilibrium with medium depends mostl on the rate at which they are cooled and their permeability to water (Mazur, 1970). If cells are cooled slowly or if their permeability to water is high, cells will equilibrate by transferring intracellular water to external ice (dehydration and shrinkage). On the other hand, if cells are cooled rapidly, or if their permeability to water is low, or even if cells are stored in LN_2 before nucleation is completed, cells will equilibrate, at least in part, by intracellular freezing.

In the present work, glycerol was found to be a better cryoprotectant than DMSO (Fig. 1). Glycerol is effective as cryoprotectant for the sperm of several species: grey mullet, Atlantic cod, *Gadus morhua* (Mounib et al., 1968), *Mugil cephalus* (Chao et al., 1975) and whitefish, *Coregonus muksun* Pallas (Piironen and Hyvarinen, 1983), although it has not been proved successfull for the rainbow trout, *Oncorhynchus mykiss* (Erdahl and Graham, 1980). Sperm motility is a good criterion for the evaluation of the effect of freezing. In the present study, it decreased after freezing in most experiments (Figs. 1-2). However, sperm motility was much better than previous reports for frozen European catfish sperm (Linhart et al., 1993).

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