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Examination of larval malformations in African catfish *Clarias gariepinus* following fertilization with cryopreserved sperm

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Abstract

In our earlier experiments on the cryopreservation of African catfish (*Clarias gariepinus*) sperm a high number of malformed individuals were found among the larvae hatched from eggs fertilized with frozenthawed sperm. In our present study we explored one of the possible reasons of this malformation by examining the ploidy of the larvae. Sperm was frozen in a 6% fructose extender containing 10% methanol and 10% DMSO as cryoprotectant. Sperm was drawn to straw of different volumes (0.25, 0.5 and 1.2 ml). Freezing was carried out in liquid nitrogen vapor. Fifteen grams or thirty grams of eggs were used for fertilization. Chromosome preparations were made of non-feeding larvae. Fifty to one hundred larvae were incubated in 0.05% colchicine for 3 h then in a hypotonic 0.075M KCl solution for 25 min. Larvae were fixed in a 3:1 solution of methanol and acetic acid, then cell suspensions were made in 50% acetic acid. The suspensions were spread on slides and stained in 4% Giemsa for 8 min. Chromosome counting and evaluation was done at 1200× magnification on well spread metaphases. A majority of the hatched larvae were diploid, with 56 chromosomes. Surprisingly some of the malformed larvae hatched from eggs fertilized with cryopreserved sperm were haploids. Haploids occurred only when 0.25 or 0.5 ml straws were used for freezing. One possible explanation of haploidy is that the genome of the fertilizing spermatozoon is damaged during the process of freezing thus it can still move and fertilize the egg but its genome does not take part in the development of the embryo. This hypothesis is supported by the fact that no haploids were found among the malformed larvae of the control group which was fertilized with fresh sperm.

Keywords: African catfish; *Clarias gariepinus*; Sperm; Cryopreservation; Chromosomes; Haploids

1. Introduction

Fish sperm cryopreservation has been extensively studied since first reported (Blaxter, 1953). Successful methods have been reported for over 200 species of

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fish and shellfish (Tiersch, 2000). Most reports have concentrated on development and comparison of new cryopreservation methods and techniques and only a few focused on the effects of cryopreservation on the frozen cell type. These include studies carried out to investigate the effect of freezing on membrane integrity or function of cellular organs of spermatozoa (Cabrita et al., 1998; Ogier de Baulny et al., 1999). To the best of our knowledge there has been only one attempt to investigate the impact of cryopreservation directly on genome integrity of spermatozoa (Labbe et al., 2001) which has been carried out using the comet assay or single cell electrophoresis on sperm DNA and the authors found that cryopreservation did not cause significant DNA damage as compared to fresh sperm.

Sperm cryopreservation techniques for African catfish (*Clarias gariepinus* Burchell, 1822) have been under development since the mid-eighties (Steyn et al., 1985; Steyn and Van Vuren, 1987a; Steyn, 1993; Urbányi et al., 1999; Viveiros et al., 2000; Ruranga et al., 2001). An effective method would be very valuable this species as the stripping of sperm is very difficult in spite of a reported successful method (Van der Waal, 1985) and thus males often have to be sacrificed and sperm extracted directly from the removed testis (Legendre et al., 1996). Thus males can be used only once for fertilisation. Large-scale cryopreservation of this species' sperm would enable the use of milt for several artificial spawnings.

In an earlier study on cryopreservation of African catfish sperm (Horváth and Urbányi, 2000) we found that hatching rate of malformed larvae in groups fertilised with frozen-thawed sperm was significantly higher than that of the control fertilised with fresh sperm. The objective of our present study was to investigate the reasons for these larval malformation by examining the ploidy of malformed larvae. A further goal of our work was to investigate the effect of freezing sperm in straws of different volumes on the fertilisation and hatching rates of African catfish eggs.

2. Materials and methods

Mature African catfish individuals (mean weight 500 g) were obtained from Szarvasfish (Szarvas, Hungary) and the Fish Culture and Irrigation Research Institute in Szarvas, Hungary. They were kept in 600 l plastic tanks at 20–22 °C with constant water filtering. The fish were fed with special pelleted catfish food (Haltáp, Szarvas, Hungary) and with frozen wild fish.

The testis of males was removed from the abdominal cavity by a simple operation, and the fish were released back to the holder tanks. The fish were anaesthetized using clove oil (10 aliquots in 10 l of water), then an approximately 20 mm long incision was made on the abdominal wall with a sterile scalpel blade and the testes were removed. The incision was closed with surgical thread. The sperm from the testes was pressed through a mesh fabric into a sterile dry Petri dish. The sperm was diluted in a 1:99 ratio in a 200 mM KCl, 30 mM Tris (pH 8.0) solution at 4 °C. Diluted sperm was activated with water in 1:9 ratio on a slide. The percentage of spermatozoa performing progressive movement (motility percentage) was observed by light microscope under 200× magnification and recorded. If the motility percentage was lower than 60% the sample was excluded from further experiments. The sperm was maintained at 4 °C until freezing.

Sperm of four males was mixed before freezing. The basic extender used in our experiments was a 6% fructose solution to which either methanol or dimethyl-sulfoxide (DMSO, both provided by Reanal, Hungary, ref. numbers: 20740-1-01-65 and 08860-1-08-65, respectively) was added as cryoprotectant in 10% V/V final concentration. After the addition of the cryoprotectant, the pH of the complete freezing medium was fixed at 7.73, which is the pH of the seminal plasma of African catfish (Steyn and Van Vuren, 1987b). $NaHCO_3$ buffer solution (1 M) was used to adjust the pH value. An equilibration time of 10 minutes was used with DMSO. The diluted sperm was drawn up into three types of straws—0.25 ml, 0.5 ml (IMV, France) and 1.2 ml (Minitüb, Germany). Freezing was done in a styrofoam box filled with liquid nitrogen. Straws were placed horizontally onto styrofoam frames floating on the surface of liquid nitrogen. For 0.5 and 1.2 ml straws the height of frames was 3 cm and the duration of freezing was 3 min. For 0.25 ml straws these parameters were 4 cm and 3 min, respectively. Following freezing sperm samples were plunged into liquid nitrogen. Straws were kept in liquid nitrogen in storage dewars