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The effects of different preservation methods on ide (*Leuciscus idus*) sperm and the longevity of sperm movement

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ABSTRACT

The present study investigated the effects of chilled storage and cryopreservation on ide sperm motility and fertilizing capacity alongside the longevity of sperm movement. The parameters of motility (progressive motility-pMOT, curvilinear velocity-VCL and straightness-STR) have been recorded during 48 h of chilled storage (4 °C) at 24-h intervals. The longevity of sperm movement was measured following activation for up to 120 s (in a range at 10–120 s) in freshly stripped and thawed sperm. A formerly established cryopreservation method was tested on ide sperm where motility parameters, hatching rate and larval malformation (according to 7 category groups) were investigated. Significant decrement of pMOT has already been observed after 24 h (6 ± 5%) compared to the freshly stripped sperm (49 ± 22%). pMOT and STR showed no significant changes for up to 120 s following activation in fresh sperm, whereas VCL showed significant difference between 10 (51 ± 11 μm/s), 90 (33 ± 3 μm/s) and 120 (31 ± 4 μm/s) seconds as well as between 20 (48 ± 12 μm/s), and 120 s. No negative effect of cryopreservation was recorded on pMOT (fresh: 49 ± 19%, cryopreserved: 22 ± 22%), VCL (fresh: 45 ± 9 μm/s and cryopreserved: 57 ± 5 μm/s), STR (fresh: 81 ± 3% and cryopreserved: 92 ± 1%) hatching rate (fresh: 22 ± 15%, cryopreserved: 33 ± 18%) or larval malformation (fresh: 12 ± 4%, cryopreserved: 12 ± 4%). No significant correlation was found between the three motility parameters and hatching rate. Cryopreservation had no effect on hatching and the prevalence of larval deformity. Furthermore craniofacial and eye deformities were characteristic in the group originating from fertilization with cryopreserved sperm, while edemas (pericardial, yolk) occurred more frequently in the control. The formerly developed cryopreservation protocol (method for cyprinids) was applicable to ide sperm.

1. Introduction

Chilled and cryogenic storage of fish sperm is a useful tool, which may efficiently support fisheries management, species conservation and research in many ways. Cryopreservation enables the synchronization of spermiation and ovulation during the spawning season, preservation of high quality gametes, establishment of gene banks for endangered species, simplification of broodstock management, and also allows to maintain cryobanks for valuable lines of model species [5,8,29].

In the last decades, species-specific chilling protocols were developed and used widely for commercially important freshwater and marine species [8,29]. Using deep freezing (−196 °C), samples can be stored even for years without quality decrease [1,40,42]. Chilled storage also allows gamete preservation for a shorter period (several hours or days). Samples need to be stored in constant conditions at an optimal temperature (which is species-specific, usually ranges between 0 and 4 °C). Another important aspect of short-term preservation of sperm is the continuous gas exchange (O_2-Co_2). The applicable chilled storage period was frequently studied in various marine and freshwater species (e.g. salmonids, sturgeons, gadids etc.) as well [5].

The evaluation of larval deformities, based on their occurrence, furthermore type might be an efficient tool to estimate the developmental potential of fish embryos. Some malformations proved to be in close relation to sperm damage following cryopreservation process [6]. Several authors studied the possible effects of freezing on the malformation of hatched larvae following thawing. Experiments usually focused on the body parameters and growth rate combined with the morphology of larvae. Many parameters (e.g. toxicity of cryoprotectants, volume of straws used for freezing and freezing process) during cryopreservation process can affect the development of hatched

Abbrevations: pMOT, progressive motility; VCL, curvilinear velocity; STR, straightness; CASA, computer assisted sperm analysis

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progeny. Horváth and Urbányi [16] found that different cryoprotectants (DMSO, DMA) affects negatively the ratio of larval deformity in African catfish (*Clarias gariepinus*). Applicability of straws with different volume (0.25, 0.5, 1.2 mL) during sperm cryopreservation of the same species, was also tested, and the malformations after hatching were estimated [30]. However, in this case cryoprotectants DMSO and methanol showed no significant effect on larval malformation rate. A similar experiment (comparison of 1.2 and 5 mL straws), where common carp (*Cyprinus carpio*) has been used as model species, found that larval malformation was not increased by cryopreservation as the highest malformation rate was recorded in the control group [18]. The deformity in the progeny following hatching was recorded in Atlantic cod (*Gadus morhua*; cryopreserved: 17–40%, control: 33–57%) [32]. Goes et al. [15] compared different artificial and natural spawning strategies (using fresh and cryopreserved sperm) in the three-barbeled catfish (*Rhamdia quelen*). Artificial propagation using cryopreserved sperm resulted in a reduction of the percentage of viable larvae.

Ide (*Leuciscus idus*) is a potamodromous, benthopelagic cyprinid species, which is native to the temperate parts of Eurasia [10,13]. The species has an important role in recreation (as gamefish) and in the ornamental fish industry. Ide was identified as a biomonitoring agent and as well as model species in toxicity experiments [23,39]. Its artificial propagation and the first attempts for domestication have already been started [23,26,27]. Experiments were conducted regarding the hormonally induced spawning process, individual sperm variability and seminal fluid composition of the species [10,12,39,41]. Optimized and successful sperm quality assessment and preservation methods can enhance the efficiency of sperm bank establishment and the hatchery practice in ide [8].

However, the information regarding chilled storage, movement longevity and cryopreservation of ide sperm are limited. Cejko et al. [11] found positive effects of an immobilizing solution and different antioxidants on ide sperm during a 17-day short-term storage (refrigerator). Sarosiek et al. [36] also revealed the positive effects of different antioxidants on sperm movement and fertilizing captivity during ide sperm cryopreservation. The sperm of the colourful orfe (colour form of ide, *Leuciscus idus* aberr *orfus*) was stored following dilution in an immobilizing solution efficiently for 5 days (4 °C) [37]. However, the chilled preservation of undiluted (fresh) sperm has not been studied yet. Targońska et al. [41] investigated the correlation between longevity (duration of sperm movement, [7]) of fresh ide sperm movement and embryo survival up to eyed-stage, however motility was investigated by visual observation only. The results indicated that sperm was able to move less than 100 s and a positive correlation was observed between sperm motility and embryonic survival. Palińska et al. [33] investigated the deformation rate in the hatched larvae obtained from controlled reproduction in ide. Poor egg quality showed notable correlation with the larval malformation rate. Overall, 11 types of deformities were identified (yolk sac deformation, cardiac edema, lordosis, kyphosis, scoliosis, axial curvature in the abdominal and caudal region, severe spine curved in axial and caudal region, C-shaped larva, pigment-deficient eye, deformed skull, body shortened). According to our knowledge, longevity of ide sperm and the larval malformation following fertilization by using thawed sperm have not been investigated yet.

Therefore the aims of our study were (1.) to experimentally measure the possible storage capacity of undiluted ide sperm; (2.) to assess the longevity of movement in fresh and thawed sperm by using CASA; and (3.) finally to test a widely used, cyprinid-based cryopreservation method [3,4,17], with regard to the hatching ratios and larvae malformations.

2. Materials and methods

2.1. Broodstock management and gamete collection

A broodstock of wild caught (from the river Zagyva) ide individuals (16 males and 9 females) was maintained at the recirculating system of the Department of Aquaculture in Szent István University, Gödöllő (Hungary). Standard length (males: 33 ± 4 cm, females: 35 ± 2 cm) and body weight (males: 747 ± 237 g, females: 1019 ± 290 g) were recorded in both sexes. The broodstock was kept in 3 m^3 plastic tank at 12 °C by using mechanical, biological and UV filtration under a 14-h daylight (low light intensity: 10 lux) and 10-h dark photoperiod. Males and females were hormonally induced only before the cryopreservation and fertilization experiment by using carp pituitary in a dose of 3.5 (males) and 5 (females) mg body weight kg^{-1} (according to T. Szabó personal communication). Rearing temperature of water was raised for 14 °C (for 2 days) prior to gamete collection. Fish were stripped 48 h following injection. Before sampling, individuals were anesthetized by using 2-phenoxyethanol (99%) at a dose of 0.4 mL/L. The genital apertures of males and females were wiped dry before the collection of gametes to prevent their activation. Sperm was hand-stripped by using 2 mL syringes. Eggs were stripped manually into dry plastic containers. Sperm samples were stored at 4 °C before the experiments (0–60 min and according to the experimental design). Eggs were stored in the hatchery at a temperature of approximately 20–25 °C for a maximum of 1 h.

2.2. Sperm analysis

Samples were activated in a Makler chamber (Sefi Medical Instruments, Haifa, Israel) by using a solution for cyprinids (45 mM NaCl, 5 mM KCl, 30 mM Tris, pH: 8.0 ± 0.2 [35]) in a mixture with 0.01 g/mL bovine serum albumin (BSA, to avoid sperm sticking). Dilution ratio (sperm: activating solution) varied according to sperm density observed during the measurement. Progressive motility (criteria according to the Sperm VisionTM v. 3.7.4.-straight line distance > 5 μm, pixel to μm ratio: 151 to 100, pMOT %), straightness (STR, %) and curvilinear velocity (VCL, μm/s) [38] of ide sperm were investigated both before (for control quality) and after cryopreservation by using a CASA (Computer-assisted Sperm Analysis) system (Sperm VisionTM v. 3.7.4., Minitube of America, Venture Court Verona, USA) [3]. Moving cells (1–100 μm^2) were identified and recorded by using a digital camera (JAI CV-A10 CL, Minitube of America, Venture Court Verona, USA) with a frame rate of 60/s.

2.3. Cryopreservation and thawing

For cryopreservation, an extender designed for salmonid species (200 mM glucose, 40 mM KCl, 30 mM Tris, pH: 8.0 ± 0.2, [19]) was prepared. Samples were first pre-diluted in grayling extender containing the cryoprotectant (10% methanol) in a ratio 1:9 and then loaded into 0.5 mL straws (Minitube GmbH, Tiefenbach, Germany). A polystyrene box filled until approximately 3 cm of depth with liquid nitrogen for freezing sperm. Diluted samples were cryopreserved 3 cm above the surface of liquid nitrogen on a floating polystyrene frame for 3 min. After 3 min, straws were plunged directly into liquid nitrogen [17]. Frozen sperm was transferred into 10-L shipping dewars (Statebourne Cryogenics, UK). Straws were thawed for 13 s in a water bath (Thermo Haake P5, Thermo Electron Corp, Waltham, Massachusetts, USA) at 40 °C. All chemicals were purchased from Reanal (Budapest, Hungary) and Sigma-Aldrich (Budapest, Hungary).

2.4. Fertilization tests

For fertilization, fresh (25 μl/group) and thawed sperm were used. Each thawed straw was divided into 2 equal portion (250 μl/group,