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Percoll gradient separation of cryopreserved common carp spermatozoa to obtain a fraction with higher motility, velocity and membrane integrity

Ping Li[a,b,*], Boris Dzyuba[a,c], Martin Hulak[a], Marek Rodina[a], Sergey Boryshpolets[a], Zhi-Hua Li[a,b], Otomar Linhart[a]

[a] *University of South Bohemia, Faculty of Fisheries and Protection of Water, Research Institute of Fish Culture and Hydrobiology, 38925 Vodnany, Czech Republic*

[b] *Key Laboratory of Freshwater Biodiversity Conservation and Utilization, Ministry of Agriculture, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Science, Jingzhou, Hubei, China*

[c] *Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavskaya str., 23, 61015 Kharkov, Ukraine*

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Abstract

We attempted to select a fraction of common carp, *Cyprinus carpio* spermatozoa that best survived a conventional freeze/thaw procedure, by centrifugation of frozen/thawed sperm through a Percoll gradient (45% and 90%). The proportion of motile spermatozoa (65.81 ± 5.19%), their velocity (77.58 ± 31.07 μm/sec), and membrane integrity (83.66 ± 4.38% intact) were significantly higher in separated sperm than in whole samples (motility 23.36 ± 2.98%, velocity 55.55 ± 19.03 μm/sec, and membrane integrity 57.92 ± 4.65%). Our results demonstrated that Percoll gradient centrifugation shows promise as a technique for selecting high quality cryopreserved fish spermatozoa, which could be useful for cryobiological research. Further studies are needed to evaluate the potentially higher fertilizing ability of the separated spermatozoa.

Keywords: *Cyprinus carpio*; cryopreservation; Percoll gradient separation; sperm; fish

1. Introduction

Cryopreservation of fish sperm may be an efficient method of preserving important genotypes, but has hitherto resulted in low spermatozoa survival and significant decrease in viability. The primary detrimental effect of cryopreservation is damage to sperm membranes, since temperature and osmotic changes cause alterations in their organization, fluidity, permeability, and lipid composition [1]. This phenomenon was also confirmed in cryopreserved sperm of halibut (*Hippoglossus hippoglossus*) which could be reactivated after adding ATP, but not on fresh sperm [2]. The freeze/thaw process consequently yields spermatozoa with low motility, fertilization ability, and larval survival, most of which are not viable [3–7]. Furthermore, dead and abnormal spermatozoa obscure the detection of the characteristics of spermatozoa showing suitable motility and intact membranes. Therefore, a procedure for separation of spermatozoa could facilitate collection of

* Corresponding author: Tel.: (420) 387 774 611; fax: (420) 387 774 634.
E-mail address: pingli06@yahoo.com (P. Li).

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high concentrations of good quality spermatozoa from frozen/thawed sperm.

For mammals, there are a number of sperm manipulation techniques available for removal of undesirable spermatozoa. These techniques include the use of a Sephadex column [8], glass wool filtration [9], swim-up methods [10], and Percoll density gradient centrifugation for separation [11]. The simplicity, reproducibility, safety, and excellent yields of motile spermatozoa through use of Percoll gradients have led to it becoming a popular technique for processing sperm of many species [12]. This technique has not been used for fish spermatozoa.

Comparisons of the efficacy of various methods of processing human cryopreserved sperm has shown Percoll gradient separation of spermatozoa to result in the recovery of spermatozoa of superior quality [13]. The aim of the present study was to assess the efficacy of Percoll gradient centrifugation to separate common carp (*Cyprinus carpio*) spermatozoa following a freeze/thaw procedure. The effect of this separation method on spermatozoa viability was evaluated by calculating the proportion of motile spermatozoa, their velocity, and their membrane integrity.

2. Materials and methods

2.1. Sperm collection and processing

The breeding and culture of common carp was carried out at the Department of Fish Genetics and Breeding, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, University of South Bohemia at Vodnany in the Czech Republic. Five males (five years old, 4–5 kg weight, and a synthetic line) were used for the experiment. Spermiation was stimulated with carp pituitary powder dissolved in 0.9% (w/v) NaCl at doses of 1 mg/kg body weight and injected 24 h prior to sperm collection. Sperm was collected in 250 mL cell culture containers and stored on ice until processing. Prior to freezing, the sperm samples were diluted 1:1 (v/v) with a cryopreservation extender (composed of 360 mg NaCl, 1000 mg KCl, 22 mg $CaCl_2$, 8 mg $MgCl_2$, and 20 mg $NaHCO_3$ in 89 mL distilled water) and 11 mL dimethyl sulfoxide (DMSO) to final volume of 100 mL (Kurokura et al [14], modified). The diluted samples were immediately loaded into 0.5 mL plastic straws (20 straws per male) and kept in liquid nitrogen vapor by floating them in a styrofoam tray (3 cm thickness) over the surface of liquid nitrogen for 20 min, followed by plunging the straws directly into liquid nitrogen. After 2 mo storage, contents were thawed by immersion of straws in a 40 °C water bath for 6 sec [15].

2.2. Sperm separation by Percoll density gradient column

A stock Percoll solution was prepared as a 9:1 mixture of Percoll and a ×10 stock salt solution (KK) comprising 3600 mg NaCl, 10 000 mg KCl, 220 mg $CaCl_2$, 80 mg $MgCl_2$, and 200 mg $NaHCO_3$ in 100 mL distilled water. The 90% Percoll solution was obtained by diluting the stock Percoll solution with KK medium. To prepare a 45% Percoll solution, the 90% Percoll solution was mixed at a 1:1 ratio with KK medium. One hundred μL of 90% Percoll solution was placed into a 1.5 mL Eppendorf tube and 100 μL of 45% Percoll was smoothly layered over this.

The 10 μL of thawed sperm suspension was gently layered onto the Percoll gradient column. Separation was performed by centrifugation at 300 × g for 10 min. After centrifugation, the supernatant above the sperm fraction was carefully removed. The bottom fraction was collected as separated samples (S). Non-separated thawed sperm was used as a control (N), diluted at a ratio of 1:5 again by ×10 dilution of KK.

2.3. Evaluation of treatment effects

Characteristics were evaluated in separated and non-separated spermatozoa by their percent motile and velocity. Motility was initiated by dilution in an activation medium (AM) containing 45 mM NaCl, 5 mM KCl, and 30 mM Tris-HCl with a pH 8.2 at a ratio of 1:2500, or by dilution in AM with 1 mM ATP (AM+ATP). To prevent spermatozoa from sticking to the microscope slide, 0.25% (w/v) pluronic (Sigma-Aldrich) was added to the solution. Motility was observed under dark-field microscopy (Olympus BX 50, Japan, objective magnification 20X) with mounted CCD video camera (SONY SSC DC50AP, Japan) and recorded using a video-DVD recorder (SONY, SVO-9500 MDP, Japan). The positions of sperm heads were captured from videosequence in five successive frames, and analyzed with a micro image analyzer (Olympus Micro Image 4.0.1. for Windows). Velocity and percentage motility were calculated from sperm head positions on five successive frames with three different colors (frame 1 red, frames 2–4 green and frame 5 blue). Spermatozoa that moved were visible in three colors, while non-moving spermatozoa were white. The percentage of motile spermatozoa was calculated from the number of white and red cells. Sperm velocity was calculated as μm/sec based on length traces of sperm from blue to green and red