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Effect of postthaw storage time and sperm-to-egg ratio on fertility of cryopreserved brook trout sperm

J. Nynca [a,*], G.J. Dietrich [a], S. Dobosz [b], T. Zalewski [b], A. Ciereszko [a]

[a] Department of Gametes and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland
[b] Department of Salmonid Research, Inland Fisheries Institute, Żukowo, Poland

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ABSTRACT

The aim of this study was to test the influence of postthaw storage time on sperm motility parameters of brook trout (n = 9). Furthermore, we examined the effect of sperm-to-egg ratios of 300,000:1 and 600,000:1 on fertility of postthaw, cryopreserved, brook trout sperm. The application of a cryopreservation procedure produced very high postthaw sperm motility (56.8 ± 4.0%). The cryopreserved sperm of brook trout could be stored up to 60 minutes without loss of the percentage of sperm motility (52.0 ± 9.0%). The fertilization capacity of brook trout postthaw sperm was comparable with the fertilization rate of fresh semen at a sperm-to-egg ratio as low as 300,000:1 (42.4 ± 14.0% and 36.5 ± 11.0% for eyed and hatched stages, respectively). The possibility of postthaw semen storage for the prolonged time and the obtainment of high fertilization rate at low sperm-to-egg ratio can lead to the significant improvement of brook trout semen cryopreservation procedure.

1. Introduction

The brook trout (*Salvelinus fontinalis*; Mitchill) of the family salmonidae is important commercially, recreationally, and ecologically in Europe. It is of interest in aquaculture because it is almost completely resistant to the viral hemorrhagic septicemia virus [1] and can easily be subjected to genome manipulation. The use of cryopreserved sperm improves the genetic characteristics of species, ensures the long-term sustainability of improved lines, reduces risks involved with the natural reproductive methods of animals, and improves efficiency in production practices. The cryopreservation procedure of brook trout semen has been evaluated [2,3]. This method secures good fertilization rates, but a high sperm-to-egg ratio (7–9 × 10^5) is necessary. Because prolonged handling time of thawed semen is of practical interest for the better organization of hatchery work, it is worth to examine how long postthaw brook trout semen can be stored without changes in sperm motility parameters. Furthermore, it is of interest if the application of lower than previously published sperm-to-egg ratios for the brook trout fertilization [2,3] will secure high fertilizing ability.

The aim of this study was to test the influence of postthaw storage time on sperm motility parameters of brook trout. Furthermore, we examined the effect of sperm-to-egg ratios of 300,000:1 and 600,000:1 on sperm motility parameters and fertilizing capacity of postthaw, cryopreserved, brook trout sperm.

2. Materials and methods

2.1. Brood stock and gamete collection

This study was approved by the Animal Experiments Local Committee in Olsztyn, Poland (no. 114/2011).

Males (n = 9; more than 2 years of age; weight, 550 ± 143 g; total length, 31.6 ± 3.2 cm) and females (n = 2; more than 3 years of age; weight, 1050 ± 211 g; total length, 38.2 ± 3.8 cm) were born and raised in the Rutki Salmonid

* Corresponding author. Tel.: +48 89 539 31 34; fax: +48 89 524 01 24.
E-mail address: j.nynca@pan.olsztyn.pl (J. Nynca).

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Research Laboratory (54.331240N; 18,341246E) at the Institute of Inland Fisheries in Olsztyn. The fish were stocked in concrete ponds (150 m^3) supplied with water from the Radunia river. The temperature of water was 6 °C to 8 °C. Oxygen saturation levels were maintained at 85% to 95%, and pH was 7.2 to 7.5. The fish were fed with a commercial diet Vitallis (Skretting, Netherlands) for spawners. Feeding was ceased 2 weeks before semen collection. Sperm and egg samples were collected at the same time at the end of the reproductive season (December 4, 2013). Before milt collection, males were anesthetized using Propiscin of 1 ppm (IFI, Żabieniec, Poland) [4]. Milt was obtained by gentle abdominal massage, with special care to avoid blood, urine, or feces contamination, and collected individually to a open glass beaker (0.5-cm depth). Samples with visible contamination were discarded. At one time, milt was collected only from three males to avoid the prolonged storage of semen (not more than 30 minutes). The average sperm concentration and osmolality of fresh milt used for cryopreservation were $7.6 \pm 1.6 \times 10^9$ spermatozoa mL^{-1} and 231 ± 27 mOsmol/kg, respectively.

2.2. Cryopreservation

Semen samples were diluted 1:5 in a 0.18-M glucose and 9% methanol extender (final concentration was 0.15 M and 7.5% for glucose and methanol, respectively). Sperm was loaded into 0.25-mL plastic straws (IMV Technologies, L'Aigle, France), placed on a 3-cm high frame made of Styrofoam, and equilibrated for 15 minutes on ice. The straws were then floated on the surface of the liquid nitrogen for 5 minutes and immersed in liquid nitrogen [5,6]. Straws were thawed in a water batch at 40 °C for 5 seconds.

2.3. Effect of postthaw sperm storage on motility parameters

Semen samples were cryopreserved as described in Section 2.2. After thawing, semen samples were stored for 60 minutes at 4 °C. Sperm motility parameters of the samples after thawing and after 60 minutes of postthaw storage at 4 °C were measured by computer-assisted sperm analysis (CASA; Hobson Vision Ltd, Baslow, UK; Section 2.5).

2.4. Effect of sperm-to-egg ratio on fertility of postthaw, cryopreserved, brook trout sperm

Sperm motility parameters were measured for fresh and frozen-thawed semen by CASA (Section 2.5). Instantly, after sperm motility measurement, semen was diluted with the extender, equilibrated, and frozen. After thawing, part of semen was immediately used for fertilization assays (within 1–2 minutes), and part from the same individual was stored for 60 minutes (Section 2.3). Fertility trials were performed with fresh and cryopreserved sperm on December 4, 2013. Eggs obtained from females by abdominal massage were mixed and divided into batches of 83 ± 7 eggs. Then, 10 mL of D532 (20-mM Tris, 30-mM glycine, and 125-mM NaCl; pH 9.0) [7] was added to the eggs together with the cryopreserved sperm. Mean ± standard deviation values of semen volumes added to the eggs were 37.0 ± 7.4 and 18.5 ± 3.7 μL at the spermatozoa-to-egg ratios of 600,000:1 and 300,000:1, respectively (the ratio was based on total spermatozoa.) Excess fresh semen (100 μL) combined from three males was used at the beginning and the end of the fertilization trials to test the quality of the eggs. All fertilization trials were done in duplicate. The fertilization rates were measured at the eyed (January 7, 2014) and hatching stages (February 3, 2014).

2.5. Semen analysis

Sperm motility parameters were measured and analyzed using the Hobson Sperm Cell Tracker (CASA; Hobson Vision Ltd) as described by Dietrich et al. [8] and Nynca et al. [6]. Sperm motility parameters were measured over a 12-second period, with a postactivation time between 5 and 17 seconds. Video recordings were analyzed using the Hobson Sperm Tracker (Hobson Vision Ltd). The program settings for the image analysis at the ×10 objective magnification were as follows: search radius = 9.69 μm; predict = off; video = pal; aspect = 1.49; refresh time = 1 second; threshold +20/−100; filter weightings 1 = 2, 2 = 2, 3 = 2, 4 = 2; and image capture rate = 50 Hz. The motile sperm were defined as a continuous analysis of the sperm head motion for a minimum of 50 points (1 second) [9]. The sperm motility parameters, such as percentage of motile sperm, straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN), and amplitude of lateral head displacement (ALH), were measured over a 12-second period, between 5 and 17 seconds postactivation time. Sperm concentration was measured using NucleoCounter SP-100 (ChemoMetec, Denmark) as described by Nynca and Ciereszko [10]. Osmolality of seminal plasma was measured using a vapor pressure osmometer 5520 (WESCOR, Logan, USA).

2.6. Statistical analysis

The data are presented as mean ± standard deviation. All analyses were performed at a significance level of 0.05. For statistical procedures, data percentages were transformed by arcsin square root transformation. The effect of time (fresh; 0 minutes postthaw and 60 minutes postthaw) on sperm motility was evaluated using one-way repeated-measures ANOVA method followed by the Tukey test for *post hoc* comparisons of means GraphPad Prism (San Diego, CA, USA). The fertilization rates of cryopreserved semen at different sperm-to-egg ratio were compared using paired *t* test. Additionally, correlation coefficients were calculated to evaluate correlations among estimated parameters.

3. Results

The percentage of postthawed sperm motility, VSL, and VAP was not affected by 60 minutes of storage, whereas a decrease in VCL and ALH and an increase in LIN were observed in cryopreserved semen (Fig. 1). The cryopreservation procedure resulted in very high postthaw sperm motility of about 57%. The percentage of fresh sperm motility decreased by 34% after cryopreservation. Similarly, a significant decline in VCL and ALH values was found for cryopreserved semen. At the same time, VSL, VAP, and LIN