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Effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout semen

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ABSTRACT

The aim of this study was to test the usefulness of 0.18 M glucose in 9% methanol as an extender, the ratio 1:5 (semen:extender) and an equilibration time of 15 min for the brown trout semen cryopreservation (n = 9). The final concentration was 0.15 M and 7.5% for glucose and methanol, respectively. Sperm motility parameters and fertilization rate at the eyed and hatching stages were assessed for post-thaw semen. The applied cryopreservation procedure resulted in remarkably high (73.8%) post-thaw sperm motility. The fertilization rate of cryopreserved brown trout semen was over 90% for sperm-to-egg ratios of both 300 000 and 600 000:1. To our knowledge, this study is the first to report the remarkably high post-thaw fertilization ability of brown trout semen at a sperm-to-egg ratio as low as 300 000:1. The described procedure for brown trout cryopreservation is highly reliable and efficient and therefore can be recommended for hatchery practice after scaling up this technique.

1. Introduction

The brown trout (*Salmo trutta* m. *fario* L.) represents a major source of freshwater fish resources in Europe because of its commercial value for aquaculture and extreme importance for angling. The brown trout is naturally subdivided into a large number of reproductively isolated and genetically distinct populations (Fergusson, 1989). The preservation of the brown trout's genetic variation is not only vital for the recovery of the original genetically unique populations but also allows selective breeding and prevents loss of fitness due to an inbreeding depression (Bartley et al., 1992).

The creation of a cryopreserved sperm bank is an effective strategy for protecting the biodiversity of the local brown trout population and provides the opportunity to preserve the sperm samples of the most valuable males, which can be used in reproductive technologies in hatchery conditions (Martínez-Páramo et al., 2009). The cryopreservation procedures of brown trout semen were evaluated using different extenders and their concentrations, dilution ratios of sperm and/or volume of straws (Dziewulska and Domagała, 2013; Lahnsteiner et al., 1997; Martínez-Páramo et al., 2009; Piironen, 1993; Sarvi et al., 2006). The fertilizing ability of cryopreserved brown trout with the described methods varied from about 45% (Dziewulska and Domagała, 2013) up to 95% (Lahnsteiner et al., 1997), but the sperm-to-egg ratio used for fertilization was very high, over 2×10^6 spermatozoa/egg (Lahnsteiner et al., 1997; Sarvi et al., 2006). This is about 10 times more than the recommended numbers for fresh semen (Billard, 1992). The high sperm-to-egg ratio used for the fertilization with cryopreserved semen limits the routine application of the method in hatchery practice.

An elaborated procedure for securing low sperm-to-egg ratio would improve the effectiveness of brown trout cryopreservation and could lead to the implementation of this procedure in hatchery conditions. Recently, an effective cryopreservation procedure with the use of glucose–methanol extender was devised for rainbow trout semen (Ciereszko et al., 2014). However, it is unknown if the described method can be used for cryopreservation of brown trout sperm. The aim of this study was to test the effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout semen. In the first experiment, the sperm motility parameters of cryopreserved brown trout semen were evaluated. In the second experiment, the post-thaw fertilization ability of brown trout semen was examined at 600 000, 300 000, 100 000 sperm-to-egg ratios.

2. Materials and methods

2.1. Collection of semen

The experiments were carried out in November (2013) on sexually matured autumn spawning brown trout (3+ years old) maintained in the Rutki Salmonid Research Laboratory at the Institute of Inland Fisheries in Olsztyn, Poland. The average weight of fish was 920 ± 184 g. The

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fish were stocked in concrete ponds (56 m^3) supplied with water from the river Radunia. The temperature of water was 6–8 °C. Oxygen saturation levels were maintained at 85–95%. Prior to milt collection, fish (n = 15) were anesthetized using Propiscin (1 ppm IFI, Żabieniec, Poland). Milt from brown trout males was obtained by abdominal massage, with special care to avoid blood, urine or feces contamination.

2.2. Cryopreservation

The cryopreservation followed the procedure as previously described (Ciereszko et al., 2014) with the use of 0.18 M glucose and 9% methanol as extender. Final concentration was 0.15 M and 7.5% for glucose and methanol, respectively. Semen was diluted with the extender at a ratio of 1:5 (semen:extender) before being drawn into 0.25-ml plastic straws (IMV Technologies, L'Agile, France). The straws with diluted semen were placed on a 3-cm high frame made of Styrofoam and equilibrated for 15 min on ice. Afterwards they floated in liquid nitrogen for 5 min and then were submerged in it. Straws were then thawed by immersion in a water bath at 40 °C for 5 s.

2.3. Sperm motility parameters of fresh and cryopreserved semen and fertilizing ability of cryopreserved semen

Semen of nine individual males was cryopreserved as described above. The motility parameters of the fresh sperm; fresh-diluted in extender equilibrated for 15 min; and frozen–thawed sperm were examined with Computer Assisted Sperm Analysis (CASA) using the Hobson Sperm Cell Tracker (Hobson Vision Ltd., Baslow, UK). Fertilization trials for cryopreserved semen were conducted as presented below (point 2.4). Analyses were run in duplicates for all of the experiments in the present study.

2.4. Fertilization ability of cryopreserved semen

Fertilization was performed on November 21, 2013. The eggs pooled from two females were divided into batches of 206 ± 15 eggs (21 g) and fertilized with thawed semen with spermatozoa to egg ratios of 600 000; 300 000 and 100 000:1. To test the quality of the eggs, excess fresh semen (50 μl) combined from three males was used at the beginning and at the end of the fertilization trial. The fertilization success was established by calculating the percentage of embryos at the eyed stage (December 24, 2013) and hatching larvae (February 17, 2014). We calculated the percentage of hatching larvae using the initial number of eggs.

2.5. Semen analysis

The motility parameters of sperm were examined with Computer Assisted Sperm Analysis using the Hobson Sperm Cell Tracker as described by Dietrich et al. (2005). Sperm was activated at a dilution ratio of 1:300 with 1 mM $CaCl_2$, 20 mM Tris, 30 mM glycine, and 125 mM NaCl, at pH 9.0 (Billard, 1992) supplemented with 0.5% bovine albumin. The sperm motility parameters: percentage of motile sperm (MOT), 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 s post activation time. Video recordings (two replicates per sample) were made using a microscope Olympus BX40 (Olympus Optical, Tokyo, Japan) with a 10× negative phase objective and a Sony CCD black and white camera (SPT-M108CE), 50 frames per second were used (Boryshpolets et al., 2013). Sperm concentration was measured in duplicate using NucleoCounter SP-100 (Chemometec, Denmark) as described by Nynca and Ciereszko (2009). Briefly, semen was diluted 100 times with a sperm immobilizing solution (100 mM NaCl, 40 mM KCl, 3 mM $CaCl_2$, 1.5 mM, $MgCl_2$ and 50 mM Tris, at pH 8.5; Morisawa and Morisawa, 1988) then 51 times with Reagent S100. Osmolality of seminal plasma and extender osmolality was measured using a Minitüb Abfüll-u Labortechnik GmbH & Co. KG apparatus (Tiefenbach, Germany).

2.6. Statistical analysis

All the results are presented as mean ± SD. All analyses were performed at a significance level of 0.05 using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). For statistical procedures data percentages were transformed by arcsin square root transformation. Data were subjected to repeated measures one-way ANOVA followed by Tukey's post hoc test.

3. Results

3.1. Sperm motility parameters of fresh and cryopreserved semen and fertilizing ability of cryopreserved semen

The percentage of sperm motility of cryopreserved semen was high, above 74%, which was significantly lower in comparison to fresh semen (90%; Fig. 1). The equilibration period of fresh semen diluted with extender for 15 min did not influence the sperm motility parameters of fresh semen. VSL and VAP declined simultaneously in cryopreserved semen by 28% and by 14% respectively, but VCL, LIN and ALH values were not affected by cryopreservation. The average sperm concentration and osmolality of fresh undiluted semen were $16.60 \pm 2.87 \times 10^9$ ml^{-1} and 271 ± 26 mOsmol/kg, respectively. The extender osmolality was 2808 mOsmol/kg.

An increased percentage of eyed embryos for the cryopreserved samples was observed with an increase in the sperm-to-egg ratio used for fertilization (Fig. 2A). The highest percentage of eyed embryos (more than 90%) was found at the spermatozoa to egg ratios of 300 000 and 600 000:1. At the 100 000:1 ratio, the fertilization ability of cryopreserved semen was significantly lower compared to 300 000 and 600 000:1 ratios, but still very high (about 87%; Fig. 2A). Very little mortality (1.2–2.1%) was observed at the hatching stage. Similarly, the highest percentage of hatched larvae was observed at 300 000 and 600 000:1 sperm-to egg ratios (Fig. 2B). The fertilization rates of eggs fertilized with an excess of fresh semen at the beginning and at the end of fertilization trial were 99.6% and 98.6%, respectively, and for the hatching stage 99.1% and 98.6%, respectively.

4. Discussion

In this study we demonstrated the usefulness of glucose–methanol extender for brown trout semen cryopreservation. Using 0.18 M glucose in 9% methanol as an extender, the ratio 1:5 (semen:extender) and an equilibration time of 15 min resulted in remarkably high (73.8%) post-thaw sperm motility. The fertilization success of cryopreserved brown trout semen was over 90% for both 300 000 and 600 000:1 sperm-to-egg ratios.

According to our knowledge, this study is the first to report a very high post-thaw fertilization ability of brown trout semen at a sperm-to-egg ratio as low as 300 000:1. Furthermore, our results demonstrate that a sperm-to-egg ratio as low as 100 000:1 also produced a very high percentage of eyed embryos. It must be emphasized that for salmonid fish a ratio 300 000:1 is recommended for fertilization with fresh semen (Billard, 1992). Therefore, the fertilizing ability of cryopreserved semen is suggested to be close to that of fresh semen. In the previous studies regarding fertilization with thawed brown trout semen, the lowest sperm-to-egg ratios ($1.2–2.4 \times 10^6$ spermatozoa per egg) assured a high post-thaw fertilization rate (95.7%) that was applied by Lahnsteiner et al. (1997). In recent studies, the sperm-to-egg ratios used in the fertilization trials with cryopreserved brown trout sperm were as high as 4×10^6 (Dziewulska and Domagała, 2013) up to 6.2×10^6 (Sarvi et al., 2006) and resulted in 45% and 66.6% fertilization rates, respectively. Summing up, our procedure decreased the sperm