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Application of glucose–methanol extender to cryopreservation of semen of sex-reversed females rainbow trout results in high post-thaw sperm motility and fertilizing ability

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ABSTRACT

We tested the usefulness of 0.18 M glucose in 9% methanol as an extender for the cryopreservation of semen of sex-reversed rainbow trout females (n = 9). The semen-to-extender ratio of 1:9 and 15 min equilibration were used. Motility and viability parameters as well as the fertilization rate at the eyed stage and hatching were assessed for post-thaw sperm. The fertilization success of cryopreserved semen was about 80% for both 500,000 and 1,000,000:1 sperm-to-egg ratios. The application of the cryopreservation procedure resulted in remarkably high post-thaw sperm motility and viability (55 and 56%, respectively). Our results for the first time show that the post-thaw fertilization ability of rainbow trout sex-reversed female semen can be similar to that of fresh semen at a sperm-to-egg ratio as low as 500,000:1. The sperm motility pattern characterized by low linearity, high amplitude of lateral head displacement and curvilinear velocity seems to be beneficial for the fertilizing ability of thawed sperm. Sperm viability appears to be a reliable indicator of the quality of thawed semen of rainbow trout sex-reversed females. The cryopreservation procedure using glucose–methanol extender after scaling up can be recommended for routine hatchery practice.

1. Introduction

All-female rainbow trout populations are desirable for aquaculture, since females reach their commercial size before becoming sexually matured and therefore food is used only for somatic growth (Donaldson, 1996). All-female populations are achieved by fertilizing eggs with homogametic (carrying X chromosome) sperm from sex-reversed females (masculinized females). Masculinization can be accomplished with hormonal treatment of embryos and larvae with androgens or their analogs. Because of this method, the females reverse to male phenotype and are able to produce spermatozoa. However, sex-reversed females usually lack spermatic ducts and therefore it is necessary to sacrifice the fish in order to collect semen (Geffen and Evans, 2000).

The semen of sex-reversed females of rainbow trout is usually of low quality and highly variable among individuals regarding maturity stage. The progress of maturation is accompanied by an increase in hydration of semen and sperm motility and decrease in sperm concentration (Nynca et al., 2012a, 2012b; Robles et al., 2003). The peak of maturity of the semen of sex-reversed females occurs later than normal males and therefore is not always synchronized with the maturity of eggs from normal females (Bromage and Cumaranatunga, 1988). Thus, a high number of sex-reversed females have to be maintained in order to secure sperm availability for artificial spawning.

Cryopreservation of semen of sex-reversed females provides several advantages to rainbow trout aquaculture. Using this technique, the best quality semen collected from sacrificed fish can be efficiently used for artificial fertilization, which would greatly facilitate breeding programs in the hatchery. Cryopreservation ensures the availability of sperm through the entire year regardless of the season and the maturity status of fish. Therefore, it is also possible to perform synchronized artificial cross-fertilizations between strains or related species which have distant periods of maturation. Cryopreservation can be also used for securing the sperm of desired strains of sex-reversed female rainbow trout in sperm banks (McAndrew et al., 1995) and transportation of semen (Lubzens et al., 1993). The development of cryopreservation techniques would therefore facilitate artificial fertilization and reduce the stock of sex-reversed females maintained at fish farms.

Cryopreserved sperm of sex-reversed female rainbow trout is characterized by low post-thaw motility (<30%; Dietrich et al., 2008) compared to semen of normal males. To compensate the low quality of frozen/thawed semen, a high sperm-to-egg ratio ($3–17 \times 10^6$) was

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used for artificial fertilization (Robles et al., 2003), while for normal males it is recommended to use sperm-to-egg ratio of 3×10^5 for fresh sperm and 3×10^6 for frozen/thawed sperm (Billard, 1992). However, these authors used DMSO as the main cryoprotectant. Recently, a simple and effective cryopreservation procedure with the use of glucose–methanol extender was established for rainbow trout semen, securing 60% post thaw motility compared to 90% in fresh semen (Ciereszko et al., 2014). However, it is unknown if the described method can be used for the cryopreservation of semen of sex-reversed females.

The aim of this study was to test the usefulness of the glucose–methanol extender for the cryopreservation of semen of sex-reversed female rainbow trout. The effect of cryopreserved semen of sex-reversed female rainbow trout on sperm motility and viability parameters and fertilizing capacity at the sperm-to-egg ratios 500,000:1 and 1,000,000:1 was investigated.

2. Materials and methods

2.1. Collection of milt and measurements of sperm concentration and seminal plasma osmolality

Three-year old sex-reversed female rainbow trout were kept at the Inland Fisheries Institute in Olsztyn, Department of Salmonid Fish Research in Rutki. The average weight of fish was 820 ± 70 g. Masculinization protocol using 11β-hydroxyandrostenedione followed that described by Kuźmiński and Dobosz (2010). Milt was obtained post mortem by cutting testes and gently squeezing through a double-layer gauze to remove any testicular tissue, during the autumn spawning on 04.12.2013. Sperm concentration was measured using the spectrophotometric method (Ciereszko and Dabrowski, 1993). Osmolality of seminal plasma was measured using a Vapor Pressure Osmometer 5520 (WESCOR, Logan, USA). Mean sperm concentration and osmolality of fresh undiluted semen were $42.8 \pm 4.2 \times 10^9$ spermatozoa and 300 ± 8 mOsm/kg, respectively.

2.2. Cryopreservation

Sperm samples (n = 9) were cryopreserved using an extender consisting of 0.18 M glucose and 9% methanol and a 15 min equilibration period (Ciereszko et al., 2014). Semen was diluted with the extender at a ratio of 1:9 (semen: extender) before being drawn into 0.25-ml plastic straws (IMV Technologies, L'Agile, France). The final concentration of glucose was 0.162 M and the final concentration of methanol was 8.1%. The straws with diluted semen were placed on a 3-cm high frame made of Styrofoam and equilibrated for 15 min on ice, followed by floating on liquid nitrogen for 5 min and then plunging into liquid nitrogen. The straws were then thawed by immersion in a water bath at 40 °C for 5 s. Crypreservation trials were run in duplicates for each sample. Sperm motility was measured for fresh, diluted–equilibrated and frozen/thawed semen. Fertilization trials and analysis of sperm viability were conducted for cryopreserved semen as described below.

2.3. Sperm motility parameters of fresh, diluted in extender and cryopreserved semen and fertilizing ability of cryopreserved semen

The motility parameters of fresh sperm, fresh-diluted in extender, sperm equilibrated for 15 min, and frozen/thawed sperm were examined with Computer Assisted Sperm Analysis (CASA) using the Hobson Sperm Tracker as described by Dietrich et al. (2005). Video recordings were made using a microscope with a 10× negative phase lense and a Sony CCD black-and-white video camera at 50 Hz frame rate. Semen was diluted a 1:500 with sperm activating solution (D532 buffer) supplemented with 0.5% bovine serum albumin to prevent the spermatozoa from sticking to the glass. After rapid mixing, 0.7 μl of this solution was immediately placed in a well of a 12-well multi-test glass slide (ICN Biomedicals Inc., Aurora, OH, USA) and cover-slipped. Sperm motility parameters were measured between 5 and 17 s post-activation. Video recordings were analyzed using the Hobson Sperm Tracker (Hobson Vision Ltd., Baslow, UK). The tracker simultaneously assessed 15 sperm motility parameters, but for simplicity only straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN = 100 × VSL/VCL), amplitude of lateral head displacement (ALH) and percentage of motile sperm were chosen for further analysis. Motility analysis was performed for fresh, fresh-diluted in extender and frozen/thawed semen. Analyses were run in duplicates for all of the experiments in the present study,

Fertilization trials for cryopreserved semen were conducted as presented below (point 2.5).

2.4. Sperm viability of frozen/thawed semen

The sperm viability of frozen/thawed spermatozoa was assessed using a supra-vital staining method and by the application of fluorophores SYBR-14 and propidium iodide (PI) (Live/Dead Sperm Viability Kit, Life Technologies TM, Eugene, OR, USA). Frozen/thawed semen samples were diluted with a sperm immobilizing solution (100 mM NaCl, 40 mM KCl, 3 mM $CaCl_2$, 1.5 mM, $MgCl_2$ and 50 mM Tris, pH 8.5, Morisawa and Morisawa, 1988) to a concentration of 30×10^6, incubated 10 min with SYBR-14 and then 5 min with PI. Immediately after the staining, the samples (at 10,000 sperm cells per sample) were analyzed using a flow cytometer FACS Aria II (Becton Dickinson, San Jose, CA, USA). Results were analyzed using FACSDiva software v. 6.1 (Becton Dickinson). Three sperm populations were distinguished regarding suitability of cell to staining with SYBR-14 and PI: live (PI− SYBR+), dying (PI+ SYBR+) and dead (PI+ SYBR−). Analyses were run in duplicates for each sample.

2.5. Fertilization

Fertilization was performed on 04.12.2013. Two sperm-to-egg ratios were tested: 500,000, and 1,000,000:1. Eggs were collected from two females and mixed together. Next, 10 ml of D532 buffer (20 mM Tris, 30 mM glycine, 125 mM NaCl, pH 9.0; Billard, 1992) were added to the eggs in a plastic 120 ml cup (11 g, 103 ± 10 eggs). The sperm sample was immediately added and the gametes were swirled. After 2 min, the eggs were rinsed with hatchery water, incubated for 5 min to water-harden the eggs and then incubated in upwelling incubation trays. The mean values (±SD) of semen volumes added to the eggs were 12.4 ± 1.2 and 24.8 ± 2.4 μl at the 500,000, and 1,000,000:1 sperm-to-egg ratios, respectively. Excess fresh semen (50 μl) combined from three males was used at the beginning and at the end of the fertilization trials to test the quality of the eggs. Fertilization rates were measured at the eyed-egg stage and hatching. Fertilization trials were run in duplicates for each sample.

2.6. Statistical analysis

The results are presented as mean ± SD. All analyses were performed at a significance level of 0.05 using GraphPad Prism software v. 6.02 (GraphPad Software Inc, San Diego, CA, USA). For statistical procedures, the data percentages were normalized by arcsin square root transformation. Data were analyzed repeated measures one-way ANOVA followed by Tukey's post hoc test. Fertilization rates were subjected to repeated measures two-way analysis of variance (ANOVA) followed by Sidak's post hoc test. The associations between parameters of semen were determined using linear regression. Analyses were run in duplicates for all of the experiments in the present study.