Aquaculture 408-409 (2013) 30–33

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Technical Note

The use of concentrated extenders to improve the efficacy of cryopreservation in whitefish spermatozoa

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ARTICLE INFO

Article history:
Received 29 March 2013
Received in revised form 8 May 2013
Accepted 16 May 2013
Available online 31 May 2013

Keywords:
Fish
Milt
Freezing
Sperm motility

ABSTRACT

The objective of this work was to test the effects of three dilutions of semen to glucose–methanol extender ratios 1:3, 1:1 and 3:1 on the sperm motility characteristics of diluted and cryopreserved whitefish spermatozoa. The final concentration of extender compounds was achieved using concentrated extenders. The application of 3:1 dilution ratio allowed for the cryopreservation of up to 3 times more spermatozoa than using the currently recommended method (dilution 1:3). However, changes in sperm motility parameters were observed in response to a lower dilution ratio of semen. Further studies should be carried out to optimize this method regarding particular steps for cryopreservation technologies, especially concerning dilution.

1. Introduction

Insufficient sperm number is of great concern in regard to the cryopreservation of fish sperm. Providing a sufficient number of cryopreserved spermatozoa for fertilization is a major challenge under practical hatchery conditions when large egg batches are used. The quality of cryopreserved sperm is low; therefore, it is recommended that the number of cryopreserved spermatozoa required for successful fertilization should be 10 times higher than for fresh semen (Billard, 1992). For this reason, large volumes of cryopreserved semen are needed to meet the conditions of fertilization in hatcheries. The cryopreservation of large volumes of semen is restricted due to nonhomogeneous and low freezing and thawing rates, and for that reason, repartition vessels of low volumes, such as straws and vials, have to be used. This creates a significant problem related to the handling of high number of vessels, especially during short-time procedures such as thawing and fertilization (Lahnsteiner et al., 2002).

A dilution of milt with an extender is a basic step for cryopreservation technology and is critical for sperm concentration in repartition vessels. For most procedures, the dilution rates of semen to extender range from 1:1 to 1:9 (Bokor et al., 2007); for salmonid fish, a ratio of 1:3 is recommended (Holtz, 1993; Lahnsteiner, 2000; Lahnsteiner et al., 1996; Legendre and Billard, 1980). In most published reports regarding dilution effects on cryopreservation success, no correction for the final concentration of cryoprotectants are reported, which means that the same extender could be used for different dilution rates. This means that final the concentration of cryoprotectants might significantly differ in relation to dilution rates, for example, the final concentration of extender compounds is 50%, 75% and 90%, for 1:1, 1:3 and 1:9 semen–extender dilution rates, respectively. This would create significantly different conditions of cryopreservation related to the concentration of extender compounds.

The objective of this work was to test the effects of three dilution semen to glucose–methanol extender ratios 1:3, 1:1 and 3:1 on the sperm motility characteristics of diluted and cryopreserved whitefish spermatozoa. Final concentrations of extender compounds were achieved using concentrated extenders.

2. Material and methods

2.1. Source of milt

The experiments were carried out on sexually mature whitefish (3+ years old) maintained in the hatchery at the Department of Salmonid Research, Inland Fisheries Institute, Rutki, Poland (Wojtczak et al., 2007). The weight and the length for these fish range from 600 to 800 g and from 37 to 41 cm, respectively. Over the course of the experiment, the fish were kept in 2 m × 3 m tanks at natural temperature and photoperiod (shortening day length). The fish were not fed during the experiment. Fish were anesthetized using Propiscin (1 ppm IFI, Zabieniec, Poland) before milt collection. Milt samples were collected by gentle abdominal massage, taking care not to contaminate them with feces, mucous or urine. In order to have enough milt for the experiment (about 2.5 ml), we formed 10 pools of semen, each pool consisted of ejaculates from two different males, so semen from 20 males was used in this study. The sperm concentration of these pools used for

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http://dx.doi.org/10.1016/j.aquaculture.2013.05.016

cryopreservation was 5.29 ± 1.58 × 10^9 spermatozoa ml^{-1}. Approval by the Animal Experiments Committee in Olsztyn, Poland, was gained before starting any of the experiments.

2.2. Extenders

Three glucose–methanol extenders for three dilution rates (semen to extender) were prepared and used for the dilution of semen (Table 1). These extenders after dilution with semen had final concentrations of 0.225 M and 7.5% for glucose and methanol, respectively.

2.3. Cryopreservation

The cryopreservation followed the procedure as previously described (Ciereszko et al., 2008; Nynca et al., 2012). The milt and the extender were stored on ice, and the milt diluted with the appropriate extender at a ratio of 1:3, 1:1 and 3:1 (Table 1), mixed and drawn into 0.25-ml plastic straws (IMV Technologies, L'Agile, France). Dilution and mixing took about 30 s. The straws (5 for each variant) were placed on a 3-cm high frame made of Styrofoam floating on liquid nitrogen for three minutes and then plunged into liquid nitrogen. Straws were then thawed two weeks later by immersion in a water bath at 40 ºC for 4 s.

2.4. Measurements of sperm motility and concentration

Sperm motility was measured in fresh semen, within 10 min after dilution and immediately (within 5 s) after thawing. The motility of the spermatozoa was measured and analyzed by Computer Assisted Sperm Analysis (CASA) using the Hobson Sperm Cell Tracker as described by Dietrich et al. (2007) and Wojtczak et al. (2007). Video recordings were made using a microscope with a 10× negative phase lens and a Sony CCD black and white video camera. Milt was first mixed at a 1:40 dilution rate with non-activating medium composed of 100 mm NaCl, 40 mm KCl, 3 mm $CaCl_2$, 1.5 mm $MgCl_2$ and 50 mm Tris and a pH of 8.5. In the second step, the semen was diluted 1:50 with sperm activating solution (1 mm $CaCl_2$, 20 mm Tris, 30 mm glycine, 125 mm NaCl, pH 9.0; Billard, 1992) supplemented with 0.2 % bovine serum albumin to prevent the spermatozoa from sticking to the glass. After rapid mixing, 0.7 μl of this solution was immediately placed into a well of a 12-well multi-test glass slide (ICN Biomedicals Inc., Aurora, OH) and covered with a cover slip. Sperm motility parameters were measured over a 15-s period, with between 5 and 20 s post-activation time. Video recordings were analyzed using the Hobson Sperm Tracker (Hobson Vision Ltd., Baslow, UK). The tracker simultaneously assessed fifteen sperm motility parameters, but for simplification, 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 (MOT) were chosen for further analysis. For fresh, diluted and cryopreserved semen, motility analysis was performed. Evaluations were made in duplicate. Sperm concentration was measured according to the method described by Ciereszko and Dabrowski (1993).

Table 1
Composition of extenders consisting of methanol and glucose for three dilution rates and volumes of milt and extenders used for cryopreservation.

Dilution rate	Glucose (M)	Methanol (%)	Milt (ml)	Extender (ml)
1:3	0.30	10	0.40	1.20
1:1	0.45	15	0.75	0.75
3:1	0.90	30	1.20	0.40

2.5. Statistical analysis

All values were expressed as mean values ± SD. The percentage data were subjected to normalization by arcsin transformation. Data were tested for normal distribution and equal variances (Bartlett's test). Data concerning sperm–extender dilution effects on fresh sperm were subjected to repeated measures one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Data concerning comparison of fresh vs. cryopreserved sperm were subjected to repeated measures two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The level of significance was set at 0.05.

3. Results

Cryopreservation caused a decrease in percentage of sperm motility, VCL and ALH (Fig. 1). However, an increase in VSL and LIN was observed, whereas VAP values were not affected. The percentage of sperm motility of fresh semen was high (87.8%) and decreased to 77.7% at 3:1 sperm–extender dilution. At the same time, the percentage of cryopreserved semen declined from 27.5% at 1:3 dilution to 21.6% at 3:1 dilution; however, this decline was not significant ($P = 0.18$). Changes in VCL were different for fresh and cryopreserved semen. For fresh semen, the highest values were observed at 1:1 dilution; and for the cryopreserved semen, the lowest values were observed for 1:1 and 3:1 dilution. Changes in VAP were similar to that of VCL and included increased values in fresh semen together with an increase in sperm–extender dilution and the opposite effect for cryopreserved semen. Both LIN and VSL of fresh semen increased along an increase of sperm–extender dilution ratio. Changes in the parameters of cryopreserved semen were not significant. A decrease in ALH values along with an increase of sperm–extender dilution ratio was found for cryopreserved but not fresh semen.

4. Discussion

In this study, we have demonstrated that using concentrated extenders can be a tool for achieving better efficacy in the cryopreservation of whitefish semen through the packing of higher numbers of spermatozoa into the same vessel. For example, under the conditions of our study, it can be estimated that at a concentration of 5.29 × 10^9 spermatozoa ml^{-1}, in one 250 μl straw 0.33 × 10^9 spermatozoa, can be packed when the sperm–extender ratio is 1:3 as currently recommended. When the ratio is increased to 3:1, 0.99 × 10^9 spermatozoa can be packed. However, some changes in sperm motility parameters were observed at the same time. A possible way to counteract these changes would be improvement of equilibration step and/or develop cryopreservation technology which is better suited to the cryopreservation of higher concentrations of spermatozoa.

Significant differences were observed between the motility parameters of fresh and cryopreserved whitefish semen maintained at Rutki station (originating from Pomerania Bay). These differences followed a pattern previously described for the autochthonic population of whitefish from Lake Łebsko, i.e. a decrease in the percentage of motility, VCL and ALH and an increase in VSL and LIN (Nynca et al., 2012). Therefore, it can be suggested that changes in sperm motility in response to a cryopreservation procedure are consistent in whitefish. It appears that the CASA method is very useful for monitoring of these changes.

In this study, we observed that the sperm parameters of fresh sperm changed in response to elevated concentrations of extender. Therefore, it is likely that these effects originated from the contact of concentrated extender with spermatozoa. A possible way to overcome this problem is to decrease the speed of extender addition (adding extender slowly in the drop manner) and increase the time of equilibration. Lahnsteiner et al. (1996) established that an equilibration of semen of up to 20 min in an extender does not affect