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Maturation of spermatozoa from rainbow trout (*Oncorhynchus mykiss*) sex-reversed females using artificial seminal plasma or glucose–methanol extender

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ABSTRACT

Masculinized females (sex-reversed females) produce only homogametic spermatozoa (X) for fertilization which is desired for the production of all-female rainbow trout populations. The milt of sex-reversed females is of low quality and must be matured through extension in maturation solutions. The aim of this study was to compare the usefulness of glucose–methanol (GM) extender with artificial seminal plasma (ASP) extender for the maturation of milt of sex-reversed female rainbow trout. Milt suspensions were incubated at 4 °C for either 15 minutes (GM extender) or 120 minutes (ASP extender). Incubation of milt diluted in either the GM or ASP extender caused a significant ($P < 0.05$) increase in the percentage of sperm motility to 76.1 ± 10.9% and 74.7 ± 18.6% for GM and ASP, respectively, but no differences between both the extenders were found. Incubation also increased the average path velocity, straight line velocity, and linearity values of spermatozoa diluted with the GM extender; at the same time, none of the other parameters changed for ASP suspensions. Sperm diluted with ASP was characterized by higher curvilinear velocity and lateral head displacement values. Percentage of eyed embryos produced by fertilization using milt diluted in the GM extender amounted to 63.6 ± 16.4% and 67.2 ± 11.9% for sperm-to-egg ratio of 300,000:1 or 600,000:1, respectively and was lower ($P < 0.05$) compared with that of ASP extender (79.5 ± 5.8% and 80.3 ± 4.7% for sperm-to-egg ratio of 300,000:1 or 600,000:1, respectively). The results of our study clearly report that the mechanism of sperm maturation by the GM extender differs from that based on ASP.

1. Introduction

Female monoculture stocks of salmonid fish, including rainbow trout (*Oncorhynchus mykiss*, Walbaum), are desired for aquaculture because of numerous advantages, such as the absence of precocious males, reduced early maturation and subsequent mortality of raised fish, and reduced number of brood stock fish [1]. The sex determination model of rainbow trout is of the XY type; to produce all-female stocks, eggs should be fertilized using X chromosome–bearing spermatozoa [2]. Such sperm can be produced from masculinized females (sex-reversed females) containing only homogametic spermatozoa (X) for fertilization. Females are masculinized through the treatment of embryos and larvae with androgens, or their analogs before sex differentiation. Masculinization impairs normal morphology of the reproductive system which leads to incomplete development of testes, and lacks or

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incomplete formation of the spermatic duct [3]. Therefore, spermatozoa must be collected directly from the testes by sacrificing the fish. For this reason, milt of sex-reversed females resembles testicular milt instead of milt of normal males. Compared with normal milt, milt of sex-reversed females is characterized by a higher sperm concentration and higher concentrations of seminal plasma proteins, but lower values of sperm motility [4,5]. These differences reflect the lower quality of testicular milt. The milt quality of sex-reversed females is quite variable which is attributed to variability in morphologic development in the testes and spermatic ducts [3].

Testicular spermatozoa of salmonid fish are characterized by no or low potential for motility; however, such potential can be acquired through the incubation of sperm suspensions in solutions mimicking seminal plasma and high pH [6]. This approach has been used for the maturing of milt from sex-reversed females. Kobayashi et al. [7] incubated the milt of sex-reversed females in artificial seminal plasma (ASP) and found that milt quality significantly improved after 2 hours of incubation at high pH. This approach apparently was also used by Robles et al. [8] who also incubated milt for 2 hours in a commercial extender to increase its quality.

During our recent study concerning the cryopreservation of milt from sex-reversed rainbow trout, we noticed that 15 minutes of equilibration of fresh milt in a simple glucose–methanol (GM) extender resulted in a substantial increase in the percentage of sperm motility by 60% [9]. This suggests that GM can be used as an alternative extender for the maturation of milt from sex-reversed rainbow trout. If successful, the GM extender could decrease the necessary maturation time from 2 hours to 15 minutes. Moreover, studies of GM effects may lay the foundation for further studies of alternative sperm activation pathways in teleost fish. The aim of this study was to compare the usefulness of the GM extender with ASP [7] for the maturation of milt of sex-reversed female rainbow trout. Sperm motility parameters and fertilizing ability were used as quality endpoints. The effect of incubation time on the efficacy of the GM extender in the stimulation of sperm motility was also tested in this study.

2. Materials and methods

2.1. Collection of milt

This study was approved by the Animal Experiments Local Committee in Olsztyn, Poland (no. 114/2011). Rainbow trout sex-reversed females (average weight, 680 ± 115 g) masculinized using 11β-hydroxyandrostenedione were kept at the Inland Fisheries Institute in Olsztyn, Department of Salmonid Research in Rutki. Masculinization protocol followed that described by Kuźmiński and Dobosz [10]. Fish from autumn spawning (December 11, 2013) were used in the experiment. Milt from sex-reversed females was obtained *post mortem* by cutting testes and gently squeezing through a double-layer gauze to remove any testicular tissue [4,5].

2.2. Effect of incubation time on sperm motility parameters of milt diluted in GM extender

Milt was collected from six sex-reversed females and kept on ice before the experiment for no more than 30 minutes. Milt (20 μL) was diluted at a ratio of 1:9 with the GM extender (180 μL). Sperm motility as described in Section 2.4.1 was recorded at 0, 5, 10, 15, and 30 minutes. The GM extender consisted of 0.18-M glucose and 9% methanol (pH 6.92).

2.3. Effect of GM extender and ASP extender on sperm motility characteristics and fertilizing ability

Milt was collected from nine sex-reversed females and diluted at the same time either with the GM extender or ASP extender consisting of 7.60 g of NaCl, 2.98 g of KCl, 0.37 g of $CaCl_2$ x $2H_2O$, 0.31 g of $MgCl_2$ x 6 H_2O, 0.21 g of $NaHCO_3$, and 1000 mL of deionized water [7]. pH of ASP extender was adjusted to 9.9 with NaOH. Milt suspensions were incubated at 4 °C for either 15 minutes (GM extender) or 120 minutes (ASP extender). At these times, sperm motility parameters were measured and fertilization trials performed. To handle the experiment, the first dilution was performed for five milt samples, and after 40 minutes, it was repeated for the next four milt samples.

2.4. Milt analysis

2.4.1. Sperm quality assessment

The motility parameters of the spermatozoa were measured and analyzed using a Hobson Sperm Tracker (Hobson Vision Ltd., Baslow, UK) as previously described [4,5,9]. Video recordings (two replicates per sample) were made using a microscope with a 10-negative-phase objective and a Sony CCD black-and-white camera (SPT-M108CE). Sperm was activated at a dilution ratio of 1:500 with 1-mM $CaCl_2$, 20-mM Tris, 30-mM glycine, 125-mM NaCl, pH 9.0 supplemented with 0.5% bovine albumin. The sperm motility parameters, such as percentage of motile sperm, straight line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), linearity (LIN), and amplitude of lateral head displacement (ALH), were measured over a 12-second (between 5 and 17 seconds) postactivation time. Sperm concentration was measured using the spectrophotometric method [11].

2.4.2. Fertilization trial

The eggs pooled from the two females were divided into batches of 99 ± 4 eggs and fertilized with a spermatozoa-to-egg ratio of 3×10^5:1 or 6×10^5:1. Ten milliliters of D532 solution (20-mM Tris, 30-mM glycine, 125-mM NaCl, pH 9.0; [12]) was used as a fertilization medium. All fertilization trials were done on in duplicate on December 11, 2013. The fertilization success was established by calculating the percentage of embryos at the eyed stage (January 9, 2014) and after hatching (January 28, 2014). The average sperm concentration of milt used for fertilization was $32.66 \pm 3.37 \times 10^9$ spermatozoa. The average volume of milt used for fertilization at a spermatozoa-to-egg ratio of 3×10^5:1 was 9.3 ± 0.9 μL and was doubled for