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Cryopreservation of rainbow trout semen using a glucose-methanol extender

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ABSTRACT

The goal of this study was to improve post-thaw quality of cryopreserved rainbow trout semen. Quality was assessed by measuring sperm motility and fertilization rates at the eyed, hatching, and swim-up larvae stages. We first tested how varying glucose concentrations in a methanol extender (0.1, 0.2, and 0.3 M, $n = 9$ different males per concentration) and different semen-extender ratios (1:3, 1:1, and 3:1, $n = 3$ males and in a separate experiment 1:3 and 1:5, $n = 6$) affected post-thaw motility. Sperm motility and fertilization rates of samples with 0.18 M glucose in 9% methanol as an extender, a 1:5 sperm-to-extender ratio, and a 15 min equilibration period then were measured at sperm-to-egg ratios of 100,000, 300,000, and 600,000:1. The optimal cryopreservation conditions were found to be 0.15 M glucose in the extender, a 15 min equilibration period, and a 1:5 semen-to-extender ratio. This combination resulted in high post-thaw motility (49.9%) and hatching rates (67.1 ± 18.7, 78.6 ± 17.0, and 84.4 ± 10.0% for sperm-to-egg ratios of 100,000, 300,000 and 600,000:1, respectively). To our knowledge, this is the first report to demonstrate that the post-thaw fertilization ability of rainbow trout semen can be similar to that of fresh semen at a sperm-to-egg ratio as low as 600,000:1.

1. Introduction

Providing a sufficient number of cryopreserved spermatozoa for fertilization is a major challenge in the fish hatchery industry. The quality of cryopreserved rainbow trout sperm is low, so the number of cryopreserved spermatozoa required for successful fertilization is about 10 times higher than that for fresh semen (Billard, 1992). Low fertilization ability of cryopreserved semen is accompanied by low post-thaw motility (Lahnsteiner et al., 1996a). The low quality of cryopreserved semen negatively impacts rainbow trout breeding efforts. Therefore, improved techniques for rainbow trout sperm cryopreservation are needed.

Cryopreservation is used to preserve sperm for later use, but sperm can be damaged by the freezing and thawing process. To mitigate damage, extenders (media used to dilute sperm) containing cryoprotectants (compounds used to protect sperm from cold and heat shock and cytotoxicity) are used. Simple extenders that contain only permeable cryoprotectants and sugars as nonpermeable cryoprotectants have been used successfully to cryopreserve salmonid fish sperm. Permeable cryoprotectants include methanol, DMSO, DMA, and glycerol at concentrations of 5–25% and nonpermeable cryoprotectants, such as sucrose or glucose, at concentrations of 0.3–0.6 M (Bozkurt et al., 2005; Ekici et al., 2012; Holtz, 1993; Lahnsteiner et al., 1996b, 1997; Mansour et al., 2006; Piironen, 1993; Sarvi et al., 2006; Tekin et al., 2003). An extender consisting of 0.3 M glucose and 10% methanol has been used successfully to cryopreserve Arctic char semen (Mansour et al., 2006) and whitefish semen (Ciereszko et al., 2008; Nynca et al., 2012). Our preliminary data suggest that such a simple extender is also effective for the cryopreservation of rainbow trout spermatozoa. However, our unpublished observations showed that post-thaw motility was low (Dietrich et al., unpublished), as is typical for cryopreserved rainbow trout spermatozoa (Lahnsteiner et al., 1996a).

Cryopreservation involves several parameters that need be fine-tuned to improve post-thaw survival. These factors include cryoprotectant type and concentration (Lahnsteiner et al., 1996a; Maisse, 1994), equilibration time (Babiak et al., 2001; Lahnsteiner et al., 1996b; Perez-Cerezales et al., 2010), and number of spermatozoa (Ciereszko et al., 2013). The aim of this study was to identify the optimal conditions (i.e., glucose concentration in the extender, equilibration time, and the sperm-to-extender ratio for semen dilution) for cryopreservation of rainbow trout semen. Sperm motility and fertilization rate were used as quantitative endpoint metrics.

2. Materials and methods

2.1. Source of milt

Milt was collected during the spring spawning in April from rainbow trout males of the Rutki strain (3 years of age). The fish were anesthetized using Propiscin (1 ppm IFI, Żabieniec, Poland) prior to milt collection. Milt samples were collected by gentle abdominal massage, taking care not to contaminate them with feces, mucus, or urine. This study

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was approved by the Animal Experiments Local Committee in Olsztyn, Poland.

2.2. Cryopreservation

Cryopreservation of semen from individual males followed previously described procedures (Ciereszko et al., 2008, 2013; Nynca et al., 2012). The milt and extender were stored on ice, and milt samples were diluted with the appropriate extender at a desired ratio (from 3:1 to 1:5, depending on the experiment), mixed, and drawn into 0.25 ml plastic straws (IMV Technologies, L'Agile, France). Dilution and mixing took about 30 s. The straws then were placed on a 3 cm high Styrofoam frame floating on liquid nitrogen for 3 min before being plunged into liquid nitrogen. Straws were then thawed 1–2 h later by immersion in a 40 °C water bath for 5 s.

2.3. Effect of glucose concentration on sperm motility parameters of fresh and cryopreserved semen

Three extenders with different glucose concentrations were prepared; they consisted of 10% methanol and 0.1, 0.2, and 0.3 M glucose, respectively. We did not use a variant without glucose, because an external cryoprotectant is needed for successful cryopreservation. Cryopreservation was performed using a 1:3 semen-to-extender dilution ratio. Therefore, the final concentrations of glucose were 0.075, 0.15, and 0.25 M, respectively, and the final concentration of methanol was 7.5% for all variants. Sperm motility was measured for fresh-diluted, and frozen-thawed semen ($n = 9$ different males) using CASA (see 2.9). Mean sperm concentration and osmolality were $9.87 \pm 2.74 \times 10^9$ spermatozoa and 235 ± 63 mOsm/kg, respectively. Based on the motility results shown in Fig. 1, the extender (producing final concentrations of 0.15 M glucose and 7.5% methanol) was used in the following experiments. Analyses were run in duplicates for all of the experiments in the present study.

2.4. Effectiveness of concentrated extenders for cryopreservation of rainbow trout spermatozoa

Three extenders were prepared (Ciereszko et al., 2013): 0.2 M glucose and 10% methanol, 0.3 M glucose and 15% methanol and 0.6 M glucose and 30% methanol. These extenders were used at semen-to-extender dilution ratios of 1:3, 1:1, and 3:1, respectively. Therefore, the final concentration of glucose and methanol in all variants was the same: 0.15 M and 7.5%, respectively. The aforementioned final concentrations were used in all of the subsequent experiments. Sperm motility was measured for fresh, diluted, and frozen-thawed semen ($n = 3$,

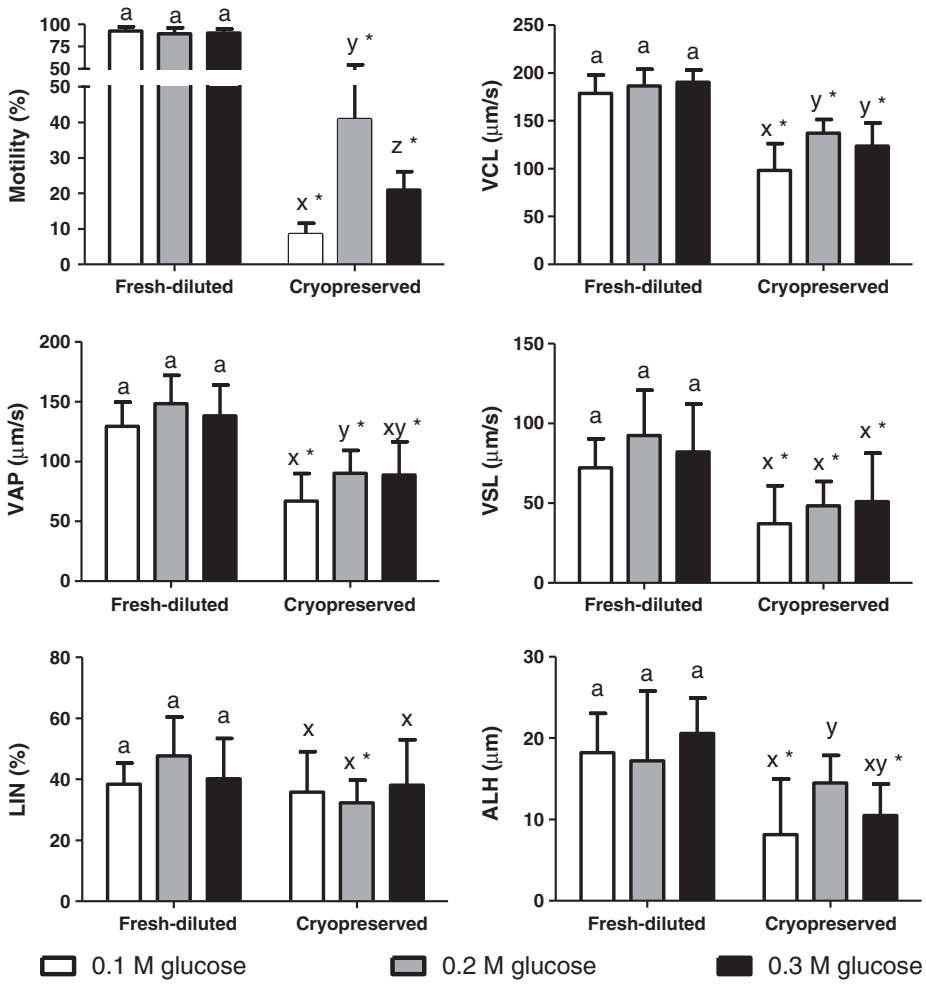

Fig. 1. Sperm motility of fresh-diluted and cryopreserved semen in relation to the concentration of glucose in the extender ($n = 9$). Results are expressed as mean ± SD. Different superscripts indicate statistical differences ($P < 0.05$) among fresh (a) and cryopreserved (x–z) semen. Asterisks indicate significant differences between the parameters for fresh and cryopreserved semen (*$P < 0.05$).