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EFFECT OF FREEZING TECHNIQUES, EXTENDERS AND CRYOPROTECTANTS ON THE FERTILIZATION RATE OF FROZEN RAINBOW TROUT *(ONCORHYNCHUS MYKISS)* SPERM

Yusuf Bozkurt[1*], Ergun Akcay[2], Necmettin Tekin[2] and Selcuk Secer[3]

[1]*Department of Aquaculture, Faculty of Fisheries, Mustafa Kemal University, 31040 Hatay, Turkey*

[2] *Department of Reproduction and Artificial Insemination, Faculty of Veterinary, Ankara University, 06110 Ankara, Turkey*

[3] *Department of Fisheries and Aquaculture, Faculty of Agriculture, Ankara University, 06110 Ankara, Turkey*

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Abstract

Rainbow trout *(Oncorhynchus mykiss)* sperm was frozen in liquid nitrogen in straws or as pellets on dry ice and stored in liquid nitrogen until artificial insemination. Sperm was diluted with one of three extenders containing 15% DMSO or 15% DMA as a cryoprotectant at a ratio of one part sperm to two parts extender. The straws were thawed in a water bath while the frozen pellets were thawed in their own extenders at 30ºC for 30 s. For both freezing methods, the best fertilization results were obtained with extender III containing 15% DMSO, 52.3% (56.3% of the control) for sperm frozen in straws and 48.4% (52.4% of the control) for sperm frozen as pellets. The interaction between extender and cryoprotectant was statistically significant ($p<0.05$).

Introduction

Cryopreserved fish sperm can be used for genetic improvement through selective breeding, production of reference stocks and hybrids, and reduction of the number of broodstock and labor involved in maintaining them. Cryopreserved sperm can help to protect stocks from total elimination due to sudden disease by allowing the introduction of new genetic lines with a reduced risk of transmitting unknown pathogens to cultured fish

* Corresponding author. E-mail: yfbozkurt@mku.edu.tr

(Lubzens et al., 1997; Chao and Liao, 2001; Akcay et al., 2002).

In spite of much progress in the field of cryopreservation of fish sperm, fertilization results vary. Development of successful techniques for the cryopreservation of fish sperm must take into account specific considerations related to the fish species such as the biochemical structure and short life span of the sperm after release into the water. Further, injuries related to freezing and thawing during cryopreservation may reduce the efficiency of various procedures. Most such injuries occur in the temperature range of 0-40ºC due to heat removal or cryoprotectant application. Other causes of cryoinjury include pH fluctuation, cold shock, ice crystal formation, osmometric effect, and cryoprotectant toxicity (Lubzens et al., 1997; Chao and Liao 2001; Akcay et al., 2004a).

Most earlier experiments in this field focussed on finding appropriate extenders and cryoprotective agents for salmonids. Generally, seminal plasma mimicking media and simple carbohydrate-based solutions are used as extenders for the cryopreservation of fish spermatozoa (Stoss and Refstie, 1983; Cognie et al., 1989; Tekin et al., 2003). Appropriate cryoprotectant solutions protect cells from cellular disruption and membrane damage during freezing and thawing (Brown and Brown, 2000; Akcay et al., 2002). Dimethyl sulfoxide (DMSO) and dimethylacetamide (DMA) have commonly been used as the internal cryoprotective agent for the cryopreservation of fish sperm (Linhart et al., 1988; Tekin et al., 2002; Akcay et al., 2004b).

Sperm diluted with extenders can be frozen using the straw or pellet method, both widely used in the aquaculture industry (Stoss and Holtz, 1983; Billard, 1992). Cryopreservation of sperm in straws reduces the time required for sperm packaging and thawing and also facilitates sperm handling during the fertilization process. Increasing attention is being paid to freezing sperm in pellets as a quick and easy freezing method.

The present study compares the straw and pellet methods using rainbow trout *(Oncorhynchus mykiss* W. 1792) sperm with the most commonly used extenders and cryoprotectants. Dry fertilization was performed to determine the success of these procedures.

Materials and Methods

Adult fish and collection of sperm. Adult rainbow trout males (2-5 years old) were obtained from the Fish Production Station (SHW) in Bolu, Turkey. In the pre-spawning period, the males were kept separately from females in small ponds and fasted 48 hours prior to sperm collection. Their abdomens and urogenital papilla were dried and sperm was collected by manual stripping. Samples contaminated with fecal material or urine were discarded. Sperm, extenders, glassware, and equipment coming into contact with sperm were kept on ice to avoid temperature shock during handling.

Evaluation of motility and dilution of sperm. Sperm motility was assessed under a microscope at 200x magnification and expressed as the mean percentage of forward-moving cells from at least three fields of view. A 0.3% NaCl solution was used as the activating solution and samples having more than 80% motile spermatozoa were selected for cryopreservation and pooled. Extenders and sperm were kept at 4ºC until dilution.

The pooled sperm was divided into 28 equal batches. Twenty-four batches were diluted at a ratio of 1:2 (sperm:extender) with one of three extenders: (I) 0.14 g $CaCl_2$, 0.2 g $MgCl_2$, 0.25 g Na_2HPO_4, 2.55 g KCl, 5.85 g NaCl, 0.1 g citric acide, 10 g glucose, 10 ml (1.27 g/100 ml) KOH, 10 ml (5.3 g/100 ml) bicine, 1 l distilled water (Erdahl and Graham, 1980); (II) 103 mM NaCl, 40 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM hepes, 1.5% BSA, 7% egg yolk, 0.5% sucrose (Lahnsteiner et al., 1998); (III) 300 mM glucose and 10% egg yolk (Tekin et al., 2003), containing either DMSO or DMA at a level of 15%. The control consisted of unfrozen and untreated sperm.

Cryopreservation by straw method. Using micropipettes, 0.5 ml plastic straws (IMV, France) were filled with the diluted sperm and sealed with polyvinile alcohol (PVA). The straws were equilibrated for 45 min at 4ºC,

then suspended on a styrofoam raft that floated 3 cm above liquid nitrogen. After 10 min, the straws were plunged into liquid nitrogen where they remained until thawing.

Cryopreservation by pellet method. Diluted sperm was rapidly dispensed into small indentations on the surface of dry ice blocks (-79ºC) and frozen within 2 min of dilution. When the frozen pellets had a frosty appearance (2-4 min), they were transferred into liquid nitrogen (-196ºC) and stored until thawing.

Thawing of sperm and fertilization. Straws were removed from the liquid nitrogen and immersed in a water bath of 30ºC for 30 s. After thawing, the straws were cut open and the sperm was immediately added to the eggs. Frozen pellets were thawed in their extender (1:10) in dry glass test tubes placed in a 30ºC water bath for 30 s. Sperm was used for fertilization within 20 s from thawing.

For fertilization, the dry fertilization technique was used at a dosage of 3 x 10^6 sperm per egg. There were four replicates of each treatment. For each replicate, sperm and 600 eggs were gently mixed about 20 s on a sterile petri dish. One min later, 25 ml of a fertilization solution (3 g urea, 4 g NaCl, 1 liter water) was added. About 45 min later, the eggs were rinsed in hatchery water. Fertilization of the untreated sperm (control) was performed similarly. The petri dishes were incubated on trays in a vertical incubator. Fertilization success was determined as the percent of eyed embryos one month later.

Statistical analysis. Results are presented as means±SE. A 2 x 2 x 3 factorial design using 3-way ANOVA was performed to analyze differences and interactions among the treatment means at a probability level of 0.05. Fertilization percentages were arcsine transformed before analysis. Paired comparisons between treatment means were performed with Tukey's test.

Results

The straw method yielded significantly higher percentages of fertilization than the pellet method for each cryoprotectant (Table 1). The highest fertilization rate was obtained in the straw method cryopreserved with DMSO. Fertilization rates significantly differed from the control. In straws, the overall means were 41.7% of the control for the DMSO treatment and 13.4% of the control for the DMA treatment. Using pellets, the overall means were 27.0% of the control for the DMSO treatment and 15.95% for the DMA treatment.

Discussion

Results of different cryoprotectants and extenders depend on factors such as fish species (freshwater or marine), freezing method (straw or pellet), and freezing procedure (equilibration, cooling, thawing, etc.). DMSO, DMA, and glycerol are the most commonly used cryoprotectants. In this study, three extenders containing DMSO or DMA were tested using two freezing procedures (straws and pellets).

The highest fertilization rate (52.3%) was obtained with sperm frozen in straws containing extender III and 15% DMSO, similar to findings reported by Conget et al. (1996) for rainbow trout (58%) and Erdahl et al. (1984) for brown trout (54%). However, Tekin et al. (2003) reported 80.5% fertility for rainbow trout and Piironen (1993) reported 79% fertility for brown trout. One reason for the difference in fertilization rates may be the volume of the straws (0.5 ml). Lahnsteiner (2000) obtained the best fertilization results using straws with a volume of ≤1.2 ml while Conget et al. (1996) obtained 58% fertility with rainbow trout sperm cryopreserved in 2.5 ml flat straws using a programmable freezer.

The highest fertilization rate with the pellet-frozen sperm (48.4%) was obtained with extender III containing 15% DMSO. This agrees with the findings of Stoss and Refstie (1983) who obtained 38.6-54.8% post-thaw fertility for sea trout containing glucose-DMSO extender. On the other hand, Babiak et al. (2001) obtained good fertilization results (76-96%) for rainbow trout when sperm was frozen with Erdahl-Graham extender containing 10% DMSO and 10% egg yolk.

The straw method for freezing rainbow trout sperm yielded considerably better fertilization rates than the pellet method. It may be that less damage is caused by the freeze-thaw

Table 1. Percent fertilization (means±SE; percent compared to control in parentheses) of rainbow trout *(Oncorhynchus mykiss)* eggs using sperm frozen in 15% dimethyl sulfoxide (DMSO) or 15% dimethyl-acetamide (DMA) and one of three extenders (n = 4).

Extender	*Straw method*	*Pellet method*
DMSO		
I	33.9±5.7^{a} (36.5)	14.3±1.0^{a} (15.5)
II	30.0±2.1^{a} (32.3)	12.1±0.2^{a} (13.1)
III	52.3±3.5^{b} (56.3)	48.4±4.5^{b} (52.4)
DMA		
I	12.7±0.8^{ab} (13.7)	14.6±0.9^{ab} (15.8)
II	11.7±0.0^{b} (12.6)	11.7±0.0^{b} (12.7)
III	12.9±0.6^{a} (13.8)	17.6±2.5^{a} (19.1)
Control	92.9±0.9^{c}	92.3±2.2^{c}

Different superscripts in a column indicate significant differences at $p<0.05$.

procedures of the straw method, but differences in the site, mechanism, and extent of damage using the both procedures should be determined. Further research is warranted on increased straw volume and frozen storage of pellets, as in other recent studies (Wheeler and Thorgard, 1991; Linhart et al., 1993). Cabrita et al. (2001) reported that dilution of the cryoprotectant causes a significant increase in fragility when the cells are exposed to hypo-osmotic shock. The extender and cryoprotectant have a higher osmolality than the sperm samples, so dilution of hypo-osmotic solutions for fertilization will cause greater osmotic stress. These conditions may have affected our fertilization results. On the other hand, optimum cryoprotectant concentrations may be influenced by fish species, sperm quality, extenders, dilution ratios, and freezing and thawing methods (Akcay et al., 2004b).

The sugar-based extender (III) resulted in a significantly higher fertilization rate than the ionic-based extenders (I and II). Sugar-based extenders have been used for the cryopreservation of sperm of African catfish *(Clarias gariepinus*; Steyn and Van Vuren, 1987; Urbanyi et al., 1999) and sturgeon species (Tsvetkova et al. 1996; Glogowski et al. 2002). Simple sugar-based extenders are reliable for cryopreservation in rainbow trout (Tekin et al., 2003). The success of sugars as extenders can be explained by their role as external cryoprotectants and membrane stabilizers (Maisse, 1996)

DMSO resulted in higher fertilization rates than DMA. Holtz (1993) obtained 80% fertility with a 0.6 M sucrose solution containing DMSO. Yamano et al. (1990) obtained 85% fertility with pellet frozen sperm using a 0.3 M glucose extender containing 10% DMSO in 50 µl pellets. Low fertilization rates were obtained when 15% DMSO or DMA was added to extenders I and II in both cryopreservation methods while the addition of DMSO or DMA to the glucose based solution (extender III) raised the fertilization rates.

The thawed spermatozoa/egg ratio in this study was 3 x 10^6. Munkittrick and Moccia (1984) reported successful fertilization rates using 3 x 10^6 thawed and 2 x 10^5 fresh sperm per egg. The lower fertility rate of thawed sperm may be due to changes in ultrastructure morphology or decreased motility and movement duration following cryopreservation. Hence, higher fertility rates for frozen sperm might be achieved by using a greater amount of spermatozoa. However, excessive sperm concentrations may mask poor sperm or egg quality and other suboptimal conditions. Increasing the efficiency of cryopreserved sperm depends on adjusting the sperm/egg ratio. The minimum sperm/egg ratio for fresh and frozen sperm for each method of cryopreservation should be investigated.

In conclusion, the present study indicates that the fertility of thawed sperm is better in straws than in pellets within the range of volumes tested. Rainbow trout sperm can be successfully cryopreserved with 15% DMSO containing a sugar-based extender. On the other hand, additional studies are necessary to evaluate the viability, survival, and development of larvae produced from cryopreserved sperm.

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