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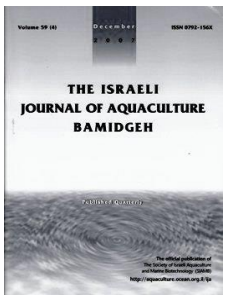

Preliminary Study on Hybridization of Brown Trout (*Salmo trutta macrostigma*) and Rainbow Trout (*Oncorhynchus mykiss*) Using Cryopreserved Sperm

Yusuf Bozkurt[1]*, İlker Yavaş[2]

[1] Mustafa Kemal University, Faculty of Marine Science and Technology, Department of Aquaculture, Hatay, Turkey

[2] Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Hatay, Turkey

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Abstract

This study examined the effect of two different extenders (carbohydrate and mineral based), supplemented with 10% Dimethyl sulfoxide (DMSO), on cryopreservation of brown trout (*Salmo trutta macrostigma*) sperm used for hybridization with rainbow trout (*Oncorhunchus mykiss*). The carbohydrate based extender resulted in higher fertilization (42.5±1.4%) than the mineral based extender and there was no difference in fertilization rates ($p>0.05$). This study successfully demonstrated that it is possible to produce *Salmo trutta macrostigma* x *Oncorhynchus mykiss* hybrids using cryopreserved sperm from brown trout.

* Corresponding author. E-mail: yfbozkurt06@gmail.com

Introduction

Hybridization is the mating of genetically differentiated individuals or groups and may involve crosses within a species or between separate species. This breeding technique is used by aquaculturists to produce aquatic organisms with specific desirable traits or enhanced performance. The desired goal is to produce offspring that perform better than either parental species. Hybridization may also be used to transfer or increase desirable characteristics from one group or species to another and to combine valuable traits from two species into a single group. It is widely used to increase growth rate, develop monosex populations and manipulate sex ratios, produce sterile fish, and to improve flesh quality, disease resistance, and environmental tolerance in aquaculture (Bartley et al., 1997). Hybridization may produce other positive traits not necessarily predictable, or even absent from the parental species.

Inter-specific hybridization has long been studied in salmonids for these purposes (Chevassus et al., 1983). However, very few of the inter-specific and inter-generic hybrids of salmonids and cyprinids have any farming or restocking potential because of their low viability or inferior performance with respect to the parental species (Purdom, 1993). Studies on the hybridization between rainbow trout (*Oncorhunchus mykiss*) and brown trout (*Salmo trutta*) found that the hybrids of these two species resulted in poor hatching rate and survival (Blanc, 2003; Blanc and Maunas, 2005).

Brown trout (*Salmo trutta macrostigma*) is a salmonid species occurring in inland water habitats of Southern Europe, Western Asia, Northern Africa, and Anatolia (Geldiay and Balik, 1988). It is a critically endangered fish species in inland waters because of illegal fishing, overfishing, and environmental changes, including pollution in general and pollution from hydroelectric plants in particular. For these reasons a biological conservation program has been considered for brown trout in Turkey. On the other hand, rainbow trout is the most cultured freshwater fish species because of its marketable properties in Turkey and for these reasons, one of the aims of this study was to produce fast growing hybrids from these two species.

The present study was designed to develop a protocol for cryopreservation of brown trout sperm and also to evaluate the possibility of hybridization between brown trout and rainbow trout using cryopreserved sperm.

Materials and Methods

Broodstock Management and Gamete Collection. Male brown trout and female rainbow trout broodfish were obtained from a private fish farm in Gaziantep, Turkey. The fish farm was supplied with fresh water collected from the bottom of an artificial lake and reoxygenated at the farm. During the experiment, broodfish were kept under a natural photoperiod in 2 m^3 fiberglass tanks supplied with recycled fresh water at 10 l/s flow rate at 9ºC.

The broodfish were not fed for 48 h prior to sperm collection and were anaesthetized with 100 ppm of MS 222 solution (Argent Labs., Redmond, WA, USA). Sperm was collected into 25 ml glass beakers by abdominal stripping from 10 brown trout males (2 years old; mean total length 45.6±1.4 cm, mean weight 984.2±45.6 g). The beakers were covered with parafilm and kept at 4°C. Eggs were collected from 5 female rainbow trout (3 years old; mean total length 42.6±0.4 cm, mean weight 1247.4±27.5 g) by gently massaging the abdomen (abdominal stripping). The abdomen and urogenital papilla of each broodfish were dried before stripping. Samples contaminated with fecal material, water, or urine were discarded. Only transparent, well rounded, smooth eggs were used for fertilization.

Sperm Quality Evaluation. Sperm motility was determined using a phase contrast microscope (Olympus, Japan) with 400X magnification. Samples were activated by mixing 1 µl of sperm with 10 µl activation solution (0.3% NaCl) on a glass slide. Motility measurements were performed within 10 seconds (s) following activation. Sperm cells that vibrated in place were not considered to be motile. Sperm motility was determined from three sample replicates. Sperm showing less than 80% motility were discarded for

cryopreservation experiments. Duration of sperm motility was determined using a sensitive chronometer (sensitivity:1/100 s) by recording the time following addition of the activation solution to the sperm samples.

Sperm density was determined according to the hemacytometric method. Sperm was diluted at a ratio of 1:1000 with Hayem solution (5g Na_2SO_4, 1g NaCl, 0.5g $HgCl_2$ 200 ml bicine) and density was determined using 100 µm deep Thoma hemocytometer (TH-100, Hecht-Assistent, Sondheim, Germany) at 400X magnification with an Olympus BX50 phase contrast microscope (Olympus, Japan) and expressed as spermatozoa $x10^9$/ml (three replicates). Counting chambers were kept moist for at least 10 min before cell counting. Sperm pH was measured using indicator papers (Merck, 5.5-9) within 10 min of sampling.

Cryopreservation Procedure. The collected sperm was stored in an ice bath and transported to the laboratory. Only spermatozoa showing vigorous movement (motility higher than 80%) were pooled and used for cryopreservation. The selected sperm samples were diluted at ratio of 1:5 with two different extenders, E1 and E2. (Table 1).

Table 1. Composition of the extenders.

Ingredients	*Extender 1*	*Extender 2*
Glucose (mm)	300	-
Egg Yolk (%)	10	-
NaCl (mm)	-	75
KCl (mm)	-	70
$CaCl_2$ (mm)	-	2
$MgSO_4$ (mm)	-	1
Tris (mm)	-	20

(Tekin et al.,2003) (Lahnsteiner et al.,2003)

These two extenders were supplemented with 10% Dimethyl sulfoxide (DMSO) as an internal cell cryoprotectant. Following 30 min. equilibration at 4ºC, the diluted sperm was packed into 0.25 ml straw (IMV, France) and sealed with polyvinyl alcohol (PVA). The straws were then placed on a floating rack in a styrofoam box on the surface of liquid nitrogen. The straws were frozen in liquid nitrogen vapor, 4 cm above the liquid nitrogen surface (about -120ºC), for 10 min. The straws were then plunged into liquid nitrogen (-196ºC) and stored until thawing. To thaw, the straws were plunged into a water bath at 30ºC for 25 s. The thawed sperm was activated using 0.3% NaCl and tested for post-thaw motility and motility duration. The thawed sperm was immediately used for fertilization experiments.

Hybridization experiments using cryopreserved sperm. Pooled eggs from five female rainbow trout were used to determine fertilization rates. The eggs were divided into nine plastic petri dishes (3 treatments, 3 replicates). Each petri dish contained 250 eggs. Egg samples were inseminated at a spermatozoa:egg ratio of 1×10^5:1 in dry petri dishes with cryopreserved sperm immediately after the straws were thawed. The control group was inseminated with fresh sperm. The eggs and sperm were mixed, and a solution containing 0.3 g urea and 0.4 g NaCl in 100 ml distilled water was used to activate the sperm. The sperm and eggs were stirred gently for 30 min, washed with hatchery water (+9ºC; 10mg/l O_2), and carefully transferred to the labeled vertical incubators with running water. Both live and dead eggs were counted in each incubator during incubation and the dead eggs were removed. When the fertilized eggs developed to the eyed stage, 100 eggs were randomly removed from the incubator and fertilization rates were calculated.

Statistical Analysis. Data are presented as means±SD. Differences between parameters were analyzed by repeated analysis of variance (ANOVA). Duncan's multiple range test was used to rank the means at a level of α=0.05. Analyses were carried out using SPSS 10 for Windows Statistical Software Package.

Results

The mean sperm volume, spermatoza motility, motility period, spermatozoa density, spermatocrit, and pH values of the 10 fresh sperm samples are shown in Table 2. It should be noted that motility and motility period of cryopreserved sperm decreased sharply compared to fresh sperm ($p<0.05$). Mean post-thaw sperm motility and motility

period results are presented in Table 3. The highest post-thaw motility, and fertilization rate, was found in 300 mm glucose containing extender 1.

Table 2. Spermatological parameters of brown trout (*Salmo trutta macrostigma*) (n=10).

Parameter	*Mean±SD*	*Min.*	*Max.*
Volume (ml)	8.9±9.3	0.9	28
Motility (%)	59.2±1.6	40	75
Motility Period (s)	42.5±6.8	30	55
Density ($x10^9$/ml)	19.6±1.2	16.9	22.9
Tot.Density($x10^9$)	174.4±5.6	150.4	203.8
Spermatocrit (%)	42.6±1.4	26	54
pH	7.3±1.4	7.1	7.5

Table 3. Effect of two different extenders combined with DMSO (10%) on frozen-thawed *Salmo trutta macrostigma* sperm Motility, motility period, and fertilization rates of the *Oncorhynchus mykiss* eggs.

Parameters	*Extender*	*Mean±SD*	*Min.*	*Max.*
Motility	E1	$50.6±1.2^a$	$45.2±0.4^a$	$54.7±1.2^a$
	E2	$45.4±1.4^a$	$40.6±0.2^a$	$50.5±1.6^a$
Motility Period	E1	$47.5±1.5^a$	$42.3±1.2^a$	$52.7±1.2^a$
	E2	$42.1±0.4^a$	$37.4±0.6^a$	$48.2±1.6^a$
Fert.Rate	E1	$45.2±1.4^a$	$37.6±0.2^a$	$52.5±1.4^a$
	E2	$40.4±1.8^a$	$35.2±0.4^a$	$47.6±1.2^a$
Control (Fresh sperm)	-	$80.2±0.6^b$	$76.5±1.2^b$	$84.2±1.6^b$

Values are means±SD of three replications. Values within a column followed by different superscripts are significantly different (P<0.05, ANOVA).

Discussion

Although other trout hybridization has been performed (Aras-Hisar et al., 2003), this study demonstrated successful hybridization between brown trout (male) and rainbow trout (female) using cryopreserved sperm. Sperm cryopreservation is a widely used method for long-term storage of fish semen (Piironen, 1993), and is an effective tool for conservation of genetic diversity in threatened and endangered species (Tiersch et al., 1998). It can also be used for genetic improvement through selective breeding programs (Mongkonpunya et al., 2000; Silverstein et al., 2006) and is also an effective method for synchronizing spawning between wild and hatchery populations when normal spawning times do not coincide (Cloud et al., 1990). No protocol works with all species of fish therefore it is necessary to develop species-specific protocols.

Selecting a suitable extender is a key factor in successful cryopreservation of fish semen. Although various extenders have been used for cryopreservation of salmonid sperm, carbohydrate based extenders supplemented with egg yolk are preferred. Carbohydrate-based extenders have been used for the sperm cryopreservation of African catfish (Steyn and Van Vuren, 1987; Urbanyi et al., 1999), sturgeon species (Tsvetkova et al., 1996; Glogowski et al., 2002) and rainbow trout (Tekin et al., 2003). The success of carbohydrates as extenders can be explained by their role as external cryoprotectants and membrane stabilizers (Maisse, 1996). Egg yolk is a valuable component in extenders for salmonid sperm cryopreservation and sperm motility (Baynes and Scott, 1987). However, the addition of egg yolk to the medium interferes with good visualization of spermatozoa during motility analysis.

DMSO, glycerol, dimethyl acetamide (DMA), ethylene glycol, and propylene glycol are widely used cryoprotectants in fish sperm cryopreservation. DMSO is most frequently used in long-term storage of fish sperm (Piironen, 1993) and has been very effective in the cryopreservation of rainbow trout, and other salmonid spermatozoa (Cloud, 2000). The present study also indicates that egg yolk and DMSO containing carbohydrate based extenders are reliable for the cryopreservation of brown trout sperm.

One of the most important indicators of the success of a freezing protocol is post-thaw motility. Brown trout spermatozoa motility was affected during cryopreservation. The best motility values and fertility results were obtained with extender 1 containing 10% DMSO. The proportion of motile cells decreased more rapidly in thawed sperm samples than in fresh ones. Movement duration was also affected. Similar results for the motility parameters of thawed spermatozoa have also been reported (Bozkurt et al., 2012).

The present study showed that fertilization rate was higher with the carbohydrate-based extender (E1) than with the mineral-based extenders (E2). Low fertilization rate obtained with frozen *Salmo trutta macrostigma* sperm can be explained by the lower spermatozoa/egg ratio. In the present study, the inseminations were carried out using $1x10^5$ thawed spermatozoa per egg. The successful fertilization of eggs with frozen sperm requires up to $3x10^6$ spermatozoa per egg (Munkittrick and Moccia, 1987; Billard, 1992). A higher concentration of spermatozoa would result in a higher number of viable spermatozoa, and may also increase the percentage of surviving spermatozoa following cryopreservation. On the other hand, evaluation of optimal spermatozoa/egg ratio is critical to determine the fertilization capacity of thawed sperm.

Results of this study suggest that our cryopreservation protocol is effective for the fertilization of rainbow trout eggs with brown trout sperm for the production of hybrids. Further studies should examine survival, growth, health, adaptation, and the possible ecological impact of the hybrids on natural populations.

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