EFFECT OF EXTENDER COMPOSITION AND CRYO-PROTECTANTS ON POST-THAW MOTILITY OF BROWN TROUT (*SALMO TRUTTA MACROSTIGMA*) SPERMATOZOA

YUSUF BOZKURT[1], İLKER YAVAŞ[2], FIKRET KARACA[2]
[1]Mustafa Kemal University, Faculty of Fisheries, Department of Aquaculture, Hatay, Turkey, [2]Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Hatay, Turkey

DELOVANJE EKSTENDERA I KRIOPROTEKTANATA NA MOTILITET SPERMATOZOIDA POTOČNE PASTRMKE (*SALMO TRUTTA MACROSTİGMA*) POSLE OTAPANJA

Abstrakt

Cilj ovog rada je identifikacija protokola zamrzavanja spermatozoida specifičnog za vrstu potočne pastrmke (*Salmo trutta macrostigma*) optimizacijom svih stadijuma tokom procedure krioprezervacije. U tom cilju su testirana dva različita ekstendera koji sadrže dva različita krioprotektanta.

U prvom stadijumu eksperimenta određen je kvalitet sperme zrelih mužjaka. Uzorci koji su pokazali >80 pokretljivosti su sakupljeni zajedno i razblaženi sa dva različita ekstendera koji su sadržali različite udele DMSO i glicerola na nivoima 10 i 15 %. Razblažena sperma je pakovana u zapreminu od 0.5 ml i ostavljena 30 min na 4 ºC. Potom je izlagana 10 min pari tečnog azota i uronjena u tečni azot. Zatim je krioprezervisana sperma otapana u vodenom kupatilu na 30ºC za 20 s da bi se odredila pokretljivost (%) i dužina pokretljivosti posle otapanja.

Uspeh zamrzavanja je procenjivan kroz motilitet sperme. Na osnovu dobijenih rezultata, pokretljivost zamrznute pa otopljene sperme je postignuta upotrebom glukoznog ekstendera sa 10 % glicerola od 40 %. S druge strane najbolje trajanje pokretljivosti od 42 s pokazali su uzorci sa Lahnsteiner ekstenderom koji je imao 10 % DMSO.

Ključne reči: *Salmo trutta macrostigma mleč, krioprezervacija, ekstender*

INTRODUCTION

Research on cryopreservation of fish sperm, with focus on cryopreservation protocols, has achieved great advances since the first successful cryopreserving of sperm in herring 50 yr ago (Blaxter 1953). It provides many benefits such as ease of global germplasm shipping and supply (Tiersch et al. 2004), selective breeding and hybridization with desirable characteristics (Kurokura et al. 1984), and conservation of genetic diversity (Ohta et al. 2001). Furthermore, a frozen sperm bank could maintain a continuous and stable supply of gametes for hatchery seed production or laboratory experimentation (Lubzens et al. 1997). Because of the advantages of this technique, fish sperm of over 200 freshwater and 40 marine species have been cryopreserved successfully (Gwo, 2000).

Cryopreservation of sperm in straws is advantageous for laboratory use such as gene banking, or small-scale commercial use, but it is impractical for largescale insemination at hatcheries. Therefore, much work remains to be done before cryopreservation of sperm can be successfully employed in large-scale application to fish species. There is a need to improve techniques on gamete storage and evaluation of sperm quality to facilitate optimization of controlled reproduction in fish (Alavi and Cosson, 2005). Successful sperm-freezing protocols depend on several factors such as sperm dilution rate, extender composition, cryoprotectant, freezing/thawing rates and handling factors (Bozkurt et al. 2005, Figiel and Tiersch 1998).

However, there is a little data on sperm quality in cnnection with cryopreservation. Motility is the most commonly used parameter to evaluate sperm quality in fishes (Billard et al. 1995). This parameter is acceptable so that spermatozoa must be motile to achieve fertilization. Sperm motility varies in vigor and duration not only among males but also within an individual male depending on its ripeness. Also, studies on most fish species showed that motility and motility of duration of semen may vary seasonally (Lahnsteiner and Patzner 1998). Therefore determining semen motility is an important component of a preservation program to prevent choosing poor quality semen prior to storage.

Furtherore, sperm motility is an important component of a cryopreservation program in order to prevent poor sperm quality sperm samples prior to freezing and to estimate the fertility of the stored sperm after thawing. On the other hand, differences in dilutents, cryoprotectants and freezing techniques make it difficult to achieve consistent estimates on the efficiency of cryopreservation procedure (Akçay et al., 2004). Most experiments in this field have focused on finding appropriate extenders and cryoprotective agents. Generally, two types of extenders have been used for the cryopreservation of fish spermatozoa such as: seminal plasma-mimicking media, and simple carbohydrate-based solutions (Ciereszko and Dabrowski, 1996).

Especially the use of an appropriate cryoprotectant solution prevents cells from cellular disruption and membrane damage during freezing and thawing. Usually dimethyl sulphoxide (DMSO) is applied as the internal cryoprotective agent, but other cryoprotectants such as dimethyl acetamide (DMA), ethylene glycol, glycerol and DMSO–glycerol mixtures are also considered as providing efficient results (Babiak et al., 2001). DMSO seems to provide salmonid sperm with the best protection and is used at concentrations between 5% and 15% (Tekin et al. 2007). In addition, glycerol has been successful for some species of fish; however, glycerol may be detrimental to salmonid sperm or may fail to provide adequate protection (Erdahl and Graham, 1980).

Salmo trutta macrostigma (known as mountain trout) is a salmonid species occurring in inland water habitats of Southern Europe, Western Asia, Northern Africa, and Anatolia (Geldiay and Balik, 1988). It is also critically endangered fish species in Turkish inland waters because of illegal fishing, overfishing, and other environmental changes, including hydroelectric plants and pollution. For this reason, *Salmo trutta macrostigma* has been considered for a biological conservation program in Turkey.

According to our knowledge, there is no information on cryopreservation of *Salmo trutta macrostigma* sperm. From this point of view, the main purpose of this study was to establish efficient method for cryopreservation of *Salmo trutta macrostigma* sperm and to compare the cryoprotective efficiency of cryoprotectants in different extenders.

MATERIALS AND METHODS

Broodstock management and sperm collection

During spawning season (November-December), mature brown trout (*Salmo trutta macrostigma*) males obtained from the farm of Directorate of Nature Conservation and National Parks, Turkey, were used for this research. In the pre-spawning period, the parenteral broodfishes were kept seperately in small ponds and fasted 48 h prior to sperm collection. For sperm collection, males were stripped by gentle abdominal massage and sperm was collected in dry plastic containers. Samples contaminated with blood, faeces, water or urine were discarded. Sperm was transported on ice to the laboratory within 15 min.

Evaluation of motility, density and pH of sperm

Sperm evaluation was carried out at the reproduction laboratory of the farm. For motility determination, 2 µL semen was activated on a glass slide with 50 µL of 4ºC sperm motility-activating solution (3‰ NaCl solution). The sperm motility rates (Table 1) were estimated within 10s following activation using a lightmicroscope (Olimpus, Japan) at x40 magnification. Motility determination was carried out in duplicate for each sperm sample. Sperm samples with a motility rating of ≥80% were pooled and used for cryopreservation. Time was recorded with a chronometer to determine motility period (s), from the time that spermatozoa starts moving until they stop or start to do local rotatory movement. All sperm manipulations were performed on ice (4ºC).

Table 1. Criteria used to assess the motility of brown trout (*Salmo trutta macrostigma*) sperm

Rating	Criteria
0 %	No motility (0%)
<10 %	Sperm cells are mostly immotile with few vibrations and <10% progressively motile sperm
10-25 %	Sperm cells are mostly immotile with few vibrations and 10–25% progressively motile sperm
25-50 %	Some sperm cells are immotile with some vibrations and 25–50% progressively motile sperm
50-75 %	Few sperm cells are immotile with some vibrations and 50–75% progressively motile sperm
75-90 %	Few sperm cells are showing vibrations with 75–90% progressively motile sperm
100 %	All sperm cells are progressively motile (100%).

Spermatozoa density was determined according to the haemacytometric method. Sperm was diluted at ratio of 1:1000 with Hayem solution (5g Na_2SO4, 1g NaCl, 0.5g $HgCl_2$ 200ml bicine) and mean spermatozoa count was calculated from three replicate samples for each fish at magnification of x40. Sperm density was expressed as $x10^9$ mL^{-1}. Counting chambers were always kept in a moist atmosphere for at least 10 min before cell counting. Sperm pH was measured using standard pH papers (Merck) within 30 min of sampling.

Extenders and cryopreservation procedure

The pooled sperm was diluted at a ratio of 1:3 (sperm:extender) with two different extenders that composing: (I) 75 mM NaCl, 70 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 20 mM Tris, 0.5% BSA, 10% egg yolk, 0.5% sucrose (Lahnsteiner et al., 1998); (II) 300 mM glucose and 10% egg yolk (Tekin et al., 2003). Each extender contained DMSO and glycerol at levels of 10 and 15%. The control consisted of unfrozen and untreated sperm.

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 30 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. For the aim of thawing, the frozen straws were removed from the liquid nitrogen and immersed in a water bath at 30ºC for 20 s.

Determination of post-thaw sperm motility

For post-thaw motility determination, 2 µL of frozen-thawed semen was activated on a glass slide with 50 µL of 4ºC sperm motility-activating solution, covered with a cover-slip and the sperm motility rating was estimated within 10s after activation using a lightmicroscope at x40 magnification according to the sperm motility rating (Table 1).

Statistical Analysis

Results are presented as means±SEM. Differences between parameters were analyzed by repeated analysis of variance (ANOVA). Significant means were subjected to a multiple comparison test (Duncan) for post-hoc comparisons at a level of α=0.05. All analyses were carried out using SPSS 10 for Windows statistical software package.

RESULTS

The spermatological properties of the semen collected from seven males are shown in Table 2. Semen volumes were rather variable and ranged from 8 to 25 ml and mean volume was 13.7±2.24 ml. Motility values were ranged from 60% to 95%. Samples that motility values were below than 80% were not used for the cryopreservation. The mean motility value of sperm samples were 79.2±5.28%.

Table 2. Spermatological parameters of fresh brown trout *(Salmo trutta macrostigma*) semen.

	Body Weight (kg)	Body Length (cm)	Volume (ml)	Motility (%)	Mot. Dur. (s)	Density ($x10^9$/ ml)	Total Density ($x10^9$)	pH
Mean Value(±)	2.27±0.42	42.4±2.84	13.7±2.24	79.2±5.28	40.0±4.50	21.7±1.41	284.2±32.6	7.07±0.07
Highest Value	4.5	55	25	95	50	28.4	430	7.5

Frozen sperm showed a significant decrease in quality compared to fresh sperm (Table 3). The motility decrease was similar for the 2 types of extenders tested and varied from 10% to 40% except for glucose extender containing 15% glycerol. Semen frozen with glucose extender containing 15% glycerol showed lowest post-thaw motility than the other samples. Semen frozen with glucose extender containing 10% glycerol had the highest post-thaw motility. Differences between the post-thaw motility values were not significant ($P<0.05$). Sperm motlity of fresh samples (control group) was determined as 80%.

Table 3. Post-thaw spermatozoa motility of brown trout (*Salmo trutta macrostigma*) semen cryopreserved with two diluents containing different ratios of cryoprotectants.

Parameter	n	Lahnsteiner's extender				Glucose extender			
		DMSO (10%)	DMSO (15%)	Glycerol (10%)	Glycerol (15%)	DMSO (10%)	DMSO (15%)	Glycerol (10%)	Glycerol (15%)
Motility (%)	3	30.4±2.27^{a}	20.2±3.46^{c}	20.8±2.18^{c}	25.8±4.57^{b}	20.2±2.46^{b}	20.6±3.79^{b}	40.8±2.37^{a}	10.6±2.59^{c}

Different superscripts indicate significant differences at $p<0.05$.

Motility durations (57 s) in fresh semen also showed significant differences with regard to the frozen spermatozoa (Table 4). It was observed that a decrease in motility duration occurred following cryopreservation. The longest post-thaw motility duration was achieved with Lahnsteiner's extender containing 10% DMSO as 42 s. Differences between the means of motility durations were significant ($P<0.05$). The interaction between the extender and cryoprotectant was significant ($F = 2.279$).

Table 4. Post-thaw spermatozoa motility durations of brown trout (*Salmo trutta macrostigma*) semen cryopreserved with two diluents containing different ratios of cryoprotectants

Parameter	n	Lahnsteiner's extender				Glucose extender			
		DMSO (10%)	DMSO (15%)	Glycerol (10%)	Glycerol (15%)	DMSO (10%)	DMSO (15%)	Glycerol (10%)	Glycerol (15%)
Motility dur. (s)	3	42^{a}	25^{d}	30^{c}	35^{b}	27^{b}	25^{b}	37^{a}	15^{c}

Different superscripts indicate significant differences at $p<0.05$.

DISCUSSION

Post-thaw motility is one of the most important indicators of the success of a freezing protocol. Examination of the effect of extender constituents and cryoprotectants of diluted sperm on the motility of frozen semen yielded some interesting results. *Salmo trutta macrostigma* spermatozoa motility was affected during cryopreservation. Post-thaw motility in salmonid spermatozoa is low (Lahnsteiner et al., 1996) due to physical shocks during freezing and thawing (Babiak et al., 2002). This situation indicates a severe damage to the most cells.

The efficacy of a cryoprotectant depends on the delicate balance between its toxicity and its capacity to protect the cells from the damage caused by freezing and thawing. In this experiment, interactions between extenders and cryoprotectants were significant. Therefore the interaction between extender constituents is an important point to take into consideration.

In Lahnsteiner's extender the best post-thaw motility was determined when 10% DMSO was used. On the other hand, in the glucose diluent, frozen-thawed *Salmo trutta macrostigma* semen had a highest sperm motility when 10% glycerol was used as cryoprotectant than semen cryopreserved using DMSO. It can be concluded that glucose can act as semen diluent, protect the sperm from osmolality damage and also has an external cryoprotectant effect (Leung and Jamieson 1991). Another reason for better protection of glycerol may due to well penetratration and leaving the cells much faster than DMSO. However, glycerol is found to be toxic when used at concentrations above 10%.

The proportion of motile cells decreased faster with time in thawed sperm samples than in fresh ones. Similar motility values were obtained with Lahnsteiner's and glucose extenders in this study. Furthermore, motility duration was also affected from this process. One extremely important aspect of cryopreservation is the appropriate choice of the extender medium. In the present study, we have used the extenders proposed by Lahnsteiner et al. (1995) and Tekin et al. (2003) due to they reported good motility after freezing/thawing.

Strong cumulative effects of type of diluent and type of cryoprotectant demonstrate the multifactorial action of the extender on spermatozoal resistance against freezing injuries. These factors may not give general information about its effect on cryopreservation success but interactions might explain differences in the usefulness of extender constituents.

In conclusion as far as we know, this is the first report on successful cryopreservation of *Salmo trutta macrostigma* sperm with regard to post-thaw motility. 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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