Cryopreservatıon of Rainbow Trout (*Oncorhynchus mykiss*) and Mirror Carp (*Cyprinus carpio*) Sperm with Glucose Based Extender

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ABSTRACT

In this study, cryopreservation of rainbow trout (*Oncorhynchus mykiss*) and mirror carp (*Cyprinus carpio*) spermatozoa was investigated. Following determination of the spermatological properties, the pooled ejaculates were diluted with glucose based extender. Diluted sperm was packaged in 0.5 ml straws and frozen in liquid nitrogen vapour (-196 °C) for 10 minutes and plunged into the liquid nitrogen. The straws were thawed in a water bath at 30 ºC for 30 seconds. In rainbow trout, the best post-thaw motility (%), post-thaw movement duration (s) and post-thaw fertilization results (%) were obtained as 57 %, 96 s. and 68.5 % while the same properties were determined as 64 %, 218 s. and 35 % in mirror carp respectively.

Key words: Rainbow trout (*Oncorhynchus mykiss*), mirror carp (*Cyprinus carpio*), sperm, extender, cryopreservation, straw, motility, fertilization

Gökkuşağı Alabalığı *(Oncorhynchus mykiss)* ve Aynalı Sazan *(Cyprinus carpio)* Spermasının Glukoz Tabanlı Sulandırıcı ile Dondurulması

ÖZET

Bu çalışmada, gökkuşağı alabalığı (*Oncorhynchus mykiss*) ve aynalı sazan (*Cyprinus carpio*) spermasının dondurulması araştırıldı. Spermatolojik özelliklerin belirlenmesinin ardından, ejakulatlar glukoz tabanlı sulandırıcı ile sulandırıldı. Sulandırılan sperma 0,5 ml'lik payetlere çekilerek sıvı azot buharında (-196 °C) 10 dakika süre ile donduruldu ve sıvı azot içerisine aktarıldı. Payetler 30 °C su banyosunda 30 saniye süre ile çözüldü. Gökkuşağı alabalığında çözülme sonu en iyi motilite (%), canlılık süresi (s) ve döl verimi yüzdesi sırasıyla % 57, 96 s. ve % 68,5 olarak elde edilirken aynı özellikler aynalı sazanda sırasıyla % 64, 218 s. ve % 35 olarak belirlendi.

Anahtar kelimeler: Gökkuşağı alabalığı (*Oncorhynchus mykiss)*, aynalı sazan (*Cyprinus carpio*), sperma, sulandırıcı, kryoprezervasyon, payet, motilite, döllenme

INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) and mirror carp (*Cyprinus carpio*) are the most important cultured freshwater fish species in Turkey consisting about 50 % of the annual aquaculture production. However, cryopreservation of their sperm has not been used in the hatcheries in spite of its countless advantages in Turkey.

Cryopreservation of spermatozoa has long been used in breeding of many animal species and is now spreading to aquaculture for the countless advantages like the reduced risk of transmitting infections, the production of hybrids with desirable characteristics, the creation of new selection lines useful for breeding and the setting up sperm banks (Akçay *et al.* 2002). Despite the progress made in sperm cryopreservation, the post-thaw results are highly variable between fish species (Scott and Baynes, 1980; Stoss, 1983). The variability between post-thaw results obtained with cryopreserved sperm is due to many factors such as quality of sperm and variations in cryopreservation technology including type of extenders, time of equilibration, freezing rate and conditions of thawing (Bozkurt *et al*. 2005a).

The basic objective of preserving spermatozoa is to reduce sperm motility during storage resulting high post-thaw motility and this result is achieved with the use of appropriate extenders. On the other hand, the suitability of extenders differs among fish species. In spite of widely usage of cryopreservation procedures for different fish species, the post-thaw results are not always satisfactory. In addition, there is a little research on cryopreservation of fish sperm at hatchery conditions and this technique has not been used by breeders in Turkey. So, the cryopreservation procedures need to be simplified in order to apply on hatchery conditions as practical.

The present study was carried out in order to determine the effect of glucose based extender on post-thaw motility, movement duration and fertility of the rainbow trout and mirror carp sperm at the hatchery conditions. For this aim, all steps of the cryopreservation procedure were simplified in order to obtain a rapid and practical species-specific protocol for easy application in the field.

MATERIALS AND METHODS

Adult Fish and Care

Adult rainbow trout and mirror carp males were obtained from a local Freshwater Fish hatchery. In the pre-spawning period the parental brood fish were kept seperately in small ponds and fasted 48 h prior to sperm collection (Seçer *et al*. 2004).

Sperm and Egg Collection

Abdomens and urogenital papillas of the adult males and females were dried before stripping. Sperm and eggs were collected from rainbow trout males and females by manual stripping respectively. In mirror carp, sperm was collected from anesthetized (0.1 g/l MS 222) males by abdominal stripping 12 h after a single injection of 2 mg/kg of carp pituitary extract (CPE) at 20-22 ºC water temperature. Eggs were collected by hand stripping for 10-12 h after a double injection of 3.5 mg/kg of CPE. The first injection, 10% (0.35 mg/kg) CPE was given for 10 h before the second (3.15 mg/kg). Only those egg batches that were well rounded and transparent were used. Eggs were stored not longer than 30 min at room temperature before the onset of the experiments. Sperm and egg samples contaminated with fecal material or urine were discarded. For all experiments, sperm from 10 males and eggs from 5 females were pooled in equal amounts (Tekin *et al*. 2003a, Bozkurt and Seçer, 2005b).

Evaluation of Sperm Quality

The sperm quality parameters including sperm volume (ml), spermatozoa motility (%), spermatozoa density ($x10^9$/ml), and spermatozoa movement duration (s) were evaluated.

Sperm Volume

The sperm was collected in glass tubes graded in millimetres and sperm volume was recorded immediately after collection.

Spermatozoa Motility

The motility of sperm in each sample was evaluated within the thirty minutes following sperm collection. About 10 µl sperm was placed on a glass microscope slide (1.0-1.2 mm depth) and 100 µl activation solution (0.3% NaCl) was added. Spermatozoa motility was observed under x200 magnification and the percentage of motil spermatozoa were assessed. Only forward movements by the spermatozoa were assessed as motil, whereas simply vibrating sperm were assessed as immobile. Observations were made at room temperature (20-23 °C) within thirty minutes of sperm collection (Seçer *et al*. 2004).

Spermatozoa Density

Spermatozoa density were determined by the haemacytometric method. Sperm was diluted (1/1000) in Hayem solution (5g Na_2SO_4, 1g NaCl, 0.5g $HgCl_2$, 200 ml bicine) and observed at a magnification of 200x and expressed as $x10^9$/ml. Counting chambers were always kept in a moist atmosphere for at least 10 min before cell counting (Seçer *et al*. 2004).

Duration of Spermatozoa Movement and Sperm pH

The duration of spermatozoa movement was assessed using a sensitive chronometer that was started simultaneously with the addition of activation solution into the sample. Sperm pH was measured with standart pH electrodes within thirty minutes of sampling (Tekin *et al*. 2003a).

Cryopreservation of Sperm

Sperm samples having more than 80% motile spermatozoa were accepted for freezing and pooled. The pooled sperm was diluted at a ratio of 1:3 (one part sperm/ three parts extender) with glucose based extenders. The following glucose based extenders were used. Rainbow trout: 300 mM glucose, 10% egg yolk (Tekin *et al.* 2003a). Mirror carp: 6 g glucose, 0.3 g NaCl, 0.05 g $NaHCO_3$ and 15% dimethyl acetamide (DMA) (Zhang and Liu, 1991).

The diluted sperm samples were loaded into 0.5 ml plastic straws (IMV, France) with micropipettes and the outer surfaces of straws were dried. One end of the straws were sealed with polyvinile alcohol (PVA). Afterwards, the samples were equilibrated for 45 min at 4 ºC until freezing. Following equilibration, the sealed straws were suspended on a styrofoam raft at 3 cm above of the liquid nitrogen. After a freezing period of 10 min the straws were plunged into liquid nitrogen where they stored until thawing (Tekin *et al*. 2003a).

Fertilization

Straws were thawed in a waterbath at 30 ºC for 30 s and cut open. Afterwards sperm was immediately transferred to the eggs. The following fertilization procedures were applied (Tekin *et al*. 2003a).

Rainbow Trout

Sperm and eggs were gently mixed about 20 s and one minute later 20 ml fertilization solution (3 g urea, 4 g NaCl in 1 l distilled water) was added. About 45 minutes later after fertilization, the eggs were rinsed in hatchery water and incubated in a vertical egg incubator. All fertilization trials were done as 3 replicates in sterile petri dishes with 500 eggs. The dry fertilization technique was used and the insemination dosage was $3x10^6$ spz/egg. Fertilization with untreated (control) sperm was performed in a similar way and at the same spermatozoa/egg ratio. Fertilization rate was determined as the percent of eyed eggs about one month later after fertilization (Bozkurt *et al*. 2005a).

Mirror Carp

Batches of eggs were inseminated with frozen and unfrozen (control group) sperm in sterile petri dishes. Fertilization trials were carried out in 3 replicates. Thawed sperm was added to the eggs and gently mixed before activation with 20 ml fertilization solution (3 g urea, 4 g NaCl in 1 l distilled water). The spermatozoa/egg ratio was about $2.5 x10^5$ spz/egg. Following fertilization, the eggs were rinsed with hatchery water and incubated in 7 l Zuger jars. Fertilization with untreated (control group) sperm was performed in a similar way and at the same spermatozoa/egg ratio. Fertilization ratios were determined as the percent of eyed eggs about three days later after fertilization (Akçay *et al*. 2004a).

Statistical Analysis

Results are expressed as mean±standart deviation. The arcsine transormation was used to the fertilization percentages before statistical analysis. All statistical analysis were carried out using SPSS 11 for windows software package (Bozkurt and Seçer, 2005b).

RESULTS

Sperm quality parameters of rainbow trout (*Oncorhynchus mykiss*) are summarized in table 1. Mean post-thaw motility, movement duration and fertility of rainbow trout (*Oncorhynchus mykiss*) sperm cryopreserved with glucose based extender were 52.0±6.2 %, 65.3±7.8 s. and 43.8±12.8 % respectively (Table 2).

Sperm quality parameters of mirror carp (*Cyprinus carpio*) are summarized in table 3. Mean post-thaw motility, movement duration and fertility of rainbow trout (*Oncorhynchus mykiss*) sperm cryopreserved with glucose based extender were 40±6.2%, 147.1±11.3 s. and 28.2±2.5 % respectively (Table 4).

Table 1. Sperm quality parameters of rainbow trout (*Oncorhynchus mykiss*) (n=10)

	Sperm volume (ml)	Spermatozoa motility (%)	Movement duration (s)	Spermatozoa density ($x10^9$/ml)	Total spermatozoa ($x10^9$)	pH
Means±S.D.	18.17±2.74	72.29±10.79	116.97±50.42	4.65±2.88	94.51±46.78	7.02±0.28
Range	15-19.9	70-85	78.3-174	1.52-7.2	44.25-136.8	6.7-7.2

Table 2. Mean (±SD) motility (%), movement durations (s) and fertility of frozen rainbow trout (*O. mykiss*) sperm (n=3)

	Post-thaw motility (%)	Post-thaw movement (s)	Fertilility (%)*
	52.0±6.2	65.3±7.8	43.8±12.8 (48.3)
Control	-	-	90.6±5.1

*The values in the parentheses represent percent of control.

Table 3. Sperm quality parameters of mirror carp (*C. carpio*) (n=10)

	Sperm volume (ml)	Spermatozoa motility (%)	Movement duration (s)	Spermatozoa density ($x10^9$/ml)	Total spermatozoa ($x10^9$)	pH
Means±S.D.	13.9±11.4	88.0±7.52	585.7±205.9	19.71±3.91	253.36±177.4	7.9±0.21
Range	1-40	70-95	315-930	12.3-24.6	18.3-591	7.5-8

Table 4. Mean (±SD) motility (%), movement durations (s) and fertility of frozen mirror carp (*C. carpio*) sperm (n=3)

	Post-thaw motility (%)	Post-thaw movement (s)	Fertility (%)*
	40.0±6.2	147.1±11.3	28.2±2.5 (33.3)
Control	-	-	84.6±2.5

*The values in the parentheses represent percent of control.

DISCUSSION

Finding on mean sperm volume was similar to some results (Geffen and Evans, 2000; Tekin *et al.* 2003a; Tekin *et al*. 2003b; Seçer *et al*. 2004) for rainbow trout but some results reported by McNiven *et al.* (1993) and Lahnsteiner *et al.* (1993) differed from the present study. In the case of mirror carp, the mean sperm volume was similar with the findings of Akçay et al. (2002; 2004a) but differed from the results of Bozkurt and Seçer (2004). The differences may be due to the age, feeding conditions and regime, environmental factors or spawning time.

The mean spermatozoa motility obtained from the present study for rainbow trout was similar to the finding of Tekin et al. (2003a) but differed from some reports (Munkittrick and Moccia, 1987; Levanduski and Cloud 1988; Seçer *et al*. 2004; Akçay *et al.* 2004b). In mirror carp, the present study agreed with the that of Akçay et al. (2002; 2004a) but not with the report of Bozkurt and Seçer (2004). Spermatozoa motility varies in vigor and duration not only between different males but also within the same individuals, depending on ripeness (Akçay *et al.* 2002). Most studies on fish species show that the duration and motility of sperm might vary on seasonally (Akçay et al. 2004a; Benau and Terner, 1980).

In the present study, spermatozoa density for rainbow trout agrees with Akçay *et al.* (2004b), but not with Tekin *et al.* (2003a), McNiven *et al.* (1993) Munkittrick and Moccia (1987), and Ciereszko and Dabrowski (1993). In mirror carp this finding was similar with the that of Akçay *et al.* (2002; 2004a) but differed from the results of Bozkurt and Seçer (2004). The differences may be due to feeding conditions, age, environmental factors, time of spawning or dilution ratio. The mean pH for both species are generally confirmed (Munkittrick and Moccia 1987; Tekin et al. 2003b; Bozkurt and Seçer 2004).

The fertilization rate for rainbow trout sperm was 48.3 % which is similar to findings of Wheeler and Thorgard (1991) and Lahnsteiner *et al.* (1997) that reported about 50 % fertility. However, these authors reported these findings with large (4.5 ml) straws. The fertilization rate for mirror carp sperm was 33 % which is similar with the finding of Lubzens et al. (1993) who reported around 40 % fertility. However, Linhart et al. (2000) reported 56 % post-thaw fertility with 10 % DMSO containing Kurokura extender while Horvath et al. (2003) obtained about 70 % fertilization rate using methanol containing glucose and fructose based extender.

As can be seen from the present study, sugar-based extenders can be used successfully in cryopreservation of rainbow trout and mirror carp spermatozoa. The reasons for the differences among the reports may be attributed to sperm collection, sperm-egg quality, extender compositions, cryopreservation procedures and insemination dosages used in studies. In the present study, the cryopreservation procedure was simplified in order to develop an easily applicable method for the aquaculturists in hatchery conditions. Another reason related with the post-thaw fertilization results may be the toxic effect of cryoprotectants on spermatozoa. Also, dilution of the cryoprotectants may cause a significant increase in fragility of the sperm cells.

Low fertilization rate obtained with frozen mirror carp sperm can be explained by the lower spermatozoa / egg ratio. In the present study, the inseminations were carried out using $2.5x10^5$ thawed spermatozoa per egg. Some authors (Munkittrick and Moccina, 1987; Billard, 1992) reported that the successful fertilization of eggs with fozen sperm requires up to $3x10^6$ spermatozoa per egg. From this point of view, higher concentration of spermatozoa should be used since a higher concentration would allow a higher number of viable spermatozoa, and may also increase the percentage of survived spermatozoa following cryopreservation. On the other hand, evaluation of optimal spermatozoa / egg ratio is critical to determine the fertilization capacity of thawed sperm.

In conclusion, low fertilization rates obtained with cryopreserved sperm indicates the changes in motility and movement duration following the cryopreservation process.

The present study also indicates that egg yolk and DMA containing sugar based extenders are reliable for the cryopreservation of rainbow trout and mirror carp sperm respectively.

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