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Semen Collection and Cryopreservation of Russian Sturgeon (*Acipenser gueldenstaedtii*) Reared in Turkey

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Özet

Türkiye'de Kültür Şartlarında Büyütülen Rus Mersin Balıklarından (*Acipenser gueldenstaedtii*) Sperm Alınması ve Muhafazası

Bu çalışmada bütün dünyada nesilleri azalan Asipenceridae familyasının Karadeniz ve ona bağlı nehirlerde yaşayan Rus mersininden kültür şartlarında sperm alınması ve cryopreservasyonu çalışılmıştır. Semen abdominal masaj ile toplanmıştır. Çalışmada spermlerin zamana bağlı hız ve hareketleri 5 kategoride değerlendirilmiştir. Soğutma 0 ile -4°C arasında 3°C/dakika ve -4 ile -10 °C arasında 5°C uygulanmıştır. Taze semenler %8 DMSO Karyoprotectantı kullanılarak 5 farklı ekstendır ile -196 °C'de sıvı azot içinde dondurulmuştur. Spermatokrite oranları %65 ile %91 arasında değişen spermlerin pH'sı ortalama 8.0 olmuştur. Dondurmadan 24 saat sonra sperm örnekleri 32°C su içinde 2 dakika düzenli çalkalanarak çözülmüş ve spermleri harektlendirmek için su ilave edilmiştir. Taze spermlerin su ile aktivasyonundan 10 dakika sonra hızları ve hareketlilikleri sırasıyla ++ ve %20 olarak belirlenmiştir. Elde ettiğimiz sonuçlar E2 ekstenderinin diğerlerinden daha iyi olduğunu göstermiş ve Mersin balıklarının semeninin soğuk muhafazasında faydalı bir ekstendir olabilir.

Anahtar Kelimeler: Karaca mersini, Semen toplama ve değerlendirme, muhafaza

Abstract

In this research, milt collection and cryopreservation with different extenders in endangered Russian sturgeon (Acipencer gueldenstaedtii) which lives Black Sea and its tributary rivers were studied. The semen was collected by abdominal massage. Sperm motility classified to five categories. Cooling were carried out as follow; 3°C/min between 0 and -4 and 5°C/min between -4 and -10. Sperm were frozen using 5 different extenders and with 8% DMSO as cyroprotectant at -196 °C in liquid nitrogen. Spermatocrite ratios range from 65% to 91%. Sperm pH was determined 8.0. After 24 hours the deep frozen sperm samples were thawed by quickly immersing them in a water bath at 32 ºC under constant shaking for about 2 min, and freshwater was added to stimulate the sperm motility. In ten minutes after activation, the velocity and motility of sperm were ++ and 20%. Our results indicate that E2 extender was better than the others and may useful extender for cryopreservation of sturgeon semen.

Keywords: Russian sturgeon, semen collection, semen evaluation, Cryopreservation

Introduction

Russian sturgeon which belongs to Acipenceridae is a diadromous fish distributed throughout the Black Sea, the Sea of Azov and the Caspian Sea and the rivers that run off into them (Vlasenko *et al*, 1988). After 1950's, Russian scientists intensively studied on artificial reproduction by means of hormone injection (Chebanov *et al*., 1998; 2004). Work on artificial propagation of sturgeon has been recently started in many countries: USA, France, Italy, Hungary, Poland, China etc.

In Turkey, sturgeon culture studies were started in 2001 (Çelikkale *et al.* 2002; Memiş *et al*., 2006). There have been many reports on the successful sperm cryopreservation of fish since Mounib (1978) began to study the sperm cryopreservation of coalfish in the 1950's.

The cyropreservation of sturgeon sperm studies were made by Burtsev and Serebryakova (1969) in Russia, and they used glycerol in concentrations of 5-14% in combination with egg yolk and sucrose (or lactose) or salts as cryoprotectant media. Mims (1991) was evaluated paddlefish milt in three treatments for chilled storage (without additives, MF; with 500 IU penicillin and 500 mg streptomycin mix in milt plasma, MP; and 0.9% saline solution, MS).

Motility of activated spermatozoa in MS was significantly longer than MF or MP treatments, and spermatozoa in MS had the longest chilled storage time. Ciereszko *et al.* (1996) used computer-assisted motility analysis (CASA) to evaluate the effect of cryopreservation and theophylline treatment on sperm motility of lake sturgeon *(Acipenser fulvescens)*.

Cryopreservation led to a decline in the percentage of motile spermatozoa, while other parameters of sperm motion, curvilinear and straight line velocities, linearity and amplitude of lateral head displacement were unchanged. Kopeika *et al*. (2000) attempted to adapt the established cryopreservation techniques for sturgeon sperm to *Acipenser sturio* L., 1758, using the sperm of a wild male.

The sperm was diluted 1:1 with media containing 56.0-76.0% tris-HCl-buffer, 14.4-24.0% dimethylsulfoxide and 9.6-20.0% egg yolk. The suspension was poured into 1.5 ml tubes, sealed and frozen in -196 °C liquid nitrogen vapour. Thawing took place in a 40 °C water bath. The motility of thawed sperm was 10-15%, whereas the motility in native sperm before cryopreservation was 50%. Lahnsteiner *et al*. (2004) investigated motility, acrosome reaction, fertility and cryobiological parameters of the semen of the sterlet, *Acipenser ruthenus* L. From the tested cryoprotectants only dimethyl sulphoxide (DMSO) and methanol provided sufficient cryoprotection. After freezing and thawing, the motility rates and swimming velocities were higher with DMSO than with methanol.

Optimal freezing conditions for sterlet semen were in the vapour of liquid nitrogen 35 cm (- 95°C to - 85°C) above its surface, the optimal thawing conditions at 25°C for 30 s. The acrosome reaction was not induced by these cryopreservation protocols.

Horváth *et al*. (2005) carried out two sets of sperm cryopreservation experiments on the shortnose sturgeon *(Acipenser brevirostrum)*. In the first set, the cryoprotectants methanol (MeOH) and dimethyl sulfoxide (DMSO) were investigated using three concentrations (5%, 10% and 15%). The highest post-thaw motility was found using 5% DMSO. In the second set, the Original Tsvetkova's extender (OT), Modified Tsvetkova's extender (MT) and modified Hanks' balanced salt solution (mHBSS) were investigated in combination with three MeOH concentrations.

The highest post-thaw motility, fertilization and hatching rates were observed with MT extender used in combination with 5% MeOH. In another set of experiments, the effects of two extenders (MT and mHBSS) and two concentrations of MeOH were investigated for sperm cryopreservation of pallid sturgeon *(Scaphyrinchus albus)*. The highest post-thaw motility was observed using MT and 10% MeOH while MT and 5% MeOH yielded the highest rates of fertilization and hatching.

They concluded that although hyperosmotic conditions of extenders and cryoprotectants result in higher post-thaw motility, they seem to reduce the fertilizing ability of the sperm.

Despite of the progress with respect to milt cryopreservation during recent decades, the results generally are highly variable and adaptation of specific techniques for each species is necessary. In Turkey, there aren't any researches on spawning and milt collection of sturgeon and their fertilization.

Our study aimed to investigate the properties of sperm of Russian sturgeon, Acipencer gueldenstaedtii, to transfer the method established for other sturgeon species, and to adapt the cryoprotectant medium to the sperm of this male, and to develop a suitable extender and cryopreserving its sperm for practical application

Materials and Methods

Broodstock

Seven years old two males weighting 10 and 12 kg reared in a sea cage (salinity 16%0) were used at the Central Fisheries Research Institute (in Trabzon, Turkey). The individuals were fed ad libitum once a day, on fresh trash fish. In the pre-spawning period, the broodfish were tagged individually with PIT-tag and transferred from sea cage to the fresh water farm on March 8th 2008. They were placed in 4000-l fiberglass tanks supplied with aerated flow-through fresh water renewed every hour under natural photoperiod and temperature conditions (ranging from 14 to 17°C), and fasted a month prior to semen collection.

Hormone treatment

The broodfish were anaesthetized with benzocaine (0.05 g/L) prior to both matura-tioncontrol and hormone implantation on April 8th 2008, and checked to find out whether they were mature or not by ultrasonography.

Powder form of Luteinizing Releasing Hormone analogue (des-Gly 10[D-Ala6]-LHRH-a) and cholesterol were mixed with ethanol in a ceramic bowl and then cacao butter was added and mixed well. A 30 mg of this prepared hormone was individually pelleted using a pellet mold. This pellet form of hormone was kept at -20°C until use. Amount of hormone dosage was accounted by Aydın (2008). A pellet form LHRH-a hormone (dosage was 0.03 mg/kg of fish weight) was implanted into the muscle near the 3th dorsal placa using a metal tube at 10:00 a.m. (01 May 2008). Single treatment was done.

Semen collection and evaluation

After hormone implantation, semen was collected by applying abdominal pressure around the urea-genital pore. To avoid any sperm contamination by urine which affects sperm quality, the urethra was catheterized and the urinary bladder was emptied by gently squeezing the fish belly. The genital region was cleaned and dried and sperm was then carefully sucked into a syringe by siphon and stored at 4 °C until use. The fresh sperm are placed in a 50 ml container in the ice box was moved to CFRI lab. Spermatocrite level was determined by centrifuging at 1000 RPM for 3 minutes and at 1500 RPM for 4 minutes.

The pH of sperm solution was measured by PH meter (Seven Multi Mettler-Toledo, Switzerland). In the laboratory, the motility of the fresh sperm was assessed immediately under a microscopy (magnification 40x10; Nikon E 400), by putting on the lam 10 µl sperm and 20 µl fresh water. During the entire experiment, the sperm motility was evaluated by using 5 categories, as fallow (Lui *et al*., 2006):

1- Drastic and extreme rapid movement (++++): the path of sperm motion was so fast that it was impossible to clearly follow individual sperm.

2- Fast movement (+++): the speed of moving sperm is very fast.

3- Slow movement (++): the speed of moving sperm is very slow.

4- Vibration (+): sperm does not move forward but its tail shows right and left vibrations.

5- Motionless (-): most sperm do not move and shows no movement.

Dilution and Crypreservation

In this study, 5 different solutions were chosen as an extender (Table 1). DMSO (dimethyl sulfoxide) was used as cyroprotectant. Before

the freezing, all equipments are cooled in the refrigerator. The ratio of semen and extender is 1:3 and DMSO rate are prepared 8% in glass vials. Sperm suspension was transferred from vial to 0.5 ml bull semen plastic tubes by syringe with a long needle. Before the freezing, suspension was waited 30 minutes for equilibrium (Mirzoyan *et al*., 2006). The tubes with sperm suspension were sealed by a lighter and a pliers. All processes were maintained at +4°C. The semen tubes were cooled from 4 to -6, -10 °C and at a rate of 3/min and 5/min, respectively. After that, those tubes were submerged at nitrogen vapour. Tubes were placed over 17 cm at 3 min, 10 cm and 2 cm at 2 min liquid nitrogen surface respectively.

The sperm samples were thawed 24 hours after the freezing by quickly immersing them in a water bath at 32 °C under constant shaking about 2 minutes (Mirzoyan *et al*., 2006). Thereafter, fresh water added in excess to stimulate the sperm motility immediately just before microscopic assessment.

Table1. Different extenders which are using in the experiment.

Extender	The component of the diluents (g/L)										
	pH	NaCl	KCl	$CaCl_2$	$MgCl_2$	NaH_2PO_4	$NaHCO_3$	Glucose	HEPES	HCl	NaOH
E_1	8.0	8.76	0.18	0.38	0.09	0.08	0.58	0.50	2.38	-	-
E_2	7.4	8.85	0.20	-	-	-	0.40	-	-	80	-
E_3	7.7	8.85	0.20	-	-	-	0.40	-	-	50	-
E_4	8.0	8.85	0.20	-	-	-	0.40	-	-	20	-
E_5	8.5	8.85	0.20	-	-	-	0.40	-	-	-	20

Ovulation was induced at 15 ºC with intramuscular injection of 30 lected into dry bowl by gentle massage of the abdomen. Stripping of eggs was repeated several times at 2-h intervals. Eggs from one female of the Russian sturgeon were used for fertilization. Aliquots of 200 eggs were fertilized with defrozen sperm. Sperm and eggs were mixed within 1 min in a Petri cup. For further development eggs were transferred to different incubation devices; vertical-flow incubator and zuger jar (volume 1 L) system modified from glass bottle supplied with UV-treated recirculated fresh water at 15°C, and supplied with running fresh water at 13.5°C.

Results

First and second fish gave totally 225 ml and 155 ml sperm, respectively. The spermatocrite rates ranged from 65% to 91%. Sperm pH ranged from 7.76 to 8.10. Russian sturgeon sperms showed high motility (90 - 100 %, ++++) just after contact with fresh water. Motility of sperm is observed as 50% and ++ 10 minutes after sperm activity (Figure 1).

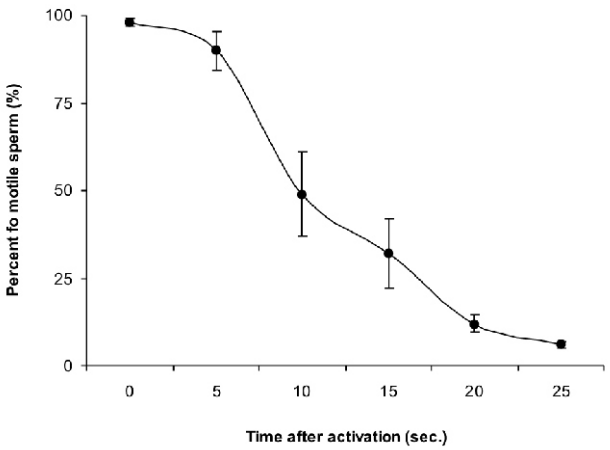

Figure 1. The motile sperm rate of *Acipenser gueldenstaedtii* semen after activation. Vertical bars indicated SD.

The sperm which was frozen with five different extenders including DMSO of 8% were thawed one day after freezing. Their motility rates were; E1, - 0%, E2, +++ 30%, E3, ++ 15%, E4, ++ 10%, E5, ++ 5%. The highest motility rate was observed with E2 extender (Table 2). Frozen thawed sperm was used for fertilization tests but there was no successful fertilization.

Discussion

The result of the current study confirmed first time sperm collection from Russian sturgeon, semen cryopreservation and post-thaw evaluation in Turkey. LHRH-a is very common in use for semen collection studies in sturgeon species (Linhart *et al*., 1995, 2006; Liu *et al*., 2006). In generally, first sperm collection takes 24 hours after the hormone injection (Glogowskia et al., 2002). The same mature male could give sperm for 5 days (Alavi personal com., 2008).

In this study, first possible stripping could be 3 days after pelleted hormone treatment this was continuing for 6 days. Hardness of LHRH-a pellet could be causing this. Measurement of spermatocrite level is very commonly used as a sperm quality aspect (Rurangwa *et al*., 2004). In this experiment, spermatocrite percentage (65% - 91%) was found higher than *Acipenser persicus* (Noveiri *et al*., 2006).

Table 2. Sperm motility rate (+ and %) in different extenders

Extenders	Sperm Motility	Motility Rate (%)
E_1	-	0
E_2	+++	30
E_3	++	15
E_4	++	10
E_5	++	5

Fish sperm cells are no motile after out of the fish body. Sperms are getting motile when they reach medium activation. After sperm activation, their survival durations are different. Generally in freshwater fish, this period is under 2 minutes (Rurangwa *et al*., 2004).

This period is up to 10 minutes for the marine fish (Dreanno *et al*., 1997). Survival period of sturgeon sperm is higher than that of the freshwater fish (Alavi *et al*., 2004).

Dimethyl-sulfoxide (DMSO), methanol and ethylene glycol were the most common cryoprotectants and sodium chloride (NaCI), potassium chloride (KCI) and saccharose in solutions buffered with Tris-HCI are used as extenders according to Billard *et al*. (2004) and Kopeika *et al*. (2007).

The different extenders are shown at Table 1. The highest post-thaw sperm motility was found in E2 (+++ %30). This motility ratio is similar with the study reported by Mirzoyan's *et al*. (2006) in which they used 15% DMSO on Russian sturgeon. Same researchers noted that fertilization rate was 55%. In this study, fertilization success was not observed due to the over-ripe egg.

188 There are not any published studies on sperm collection and cryopreservation or seed production of sturgeon in Turkey. However, there were a few studies on growth performance of Russian sturgeon (Çelikkale *et al*., 2002; Çelikkale et al., 2005; Memiş *et al*., 2006; Memiş *et al*., 2007).

At the end of the study, post-thaw sperm motility with E2 was determined as the highest percent (30%). Our results showed that post-thaw motility values were decreased in the extenders which have high Ph levels (7.7, 8.0, 8.5) (Table 2). The results of this study have shown similarity to Mirzoyan *et al*. (2006).

In conclusion, the present study indicates that Russian sturgeon semen can be successfully cryopreserved with extender E2 by adding 8% DMSO. Water bath with 32 °C for 2 min was suitable thawing condition for this species.

This protocol should improve broodstock management techniques for this species and consequently augment the potential for its culture. It should need more studies to get successful seed production in future.

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