SHORT COMMUNICATION

CRYOPRESERVATION OF RAINBOW TROUT *(ONCORHYNCHUS MYKISS)* SEMEN

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

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

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

(Received 6.12.02, Accepted 9.7.03)

Key words: cryopreservation, extender, fertilization, *Oncorhynchus mykiss*, semen, spermatological properties

Abstract

Rainbow trout semen was diluted in one of three extenders (1:3 v/v) containing egg yolk (7-10%) and 10% DMSO as cryoprotectants. The diluted semen was packaged in 0.5 ml straws and frozen in liquid nitrogen (LN_2) vapor. For thawing, the straws were immersed in a water bath for 30 sec at 30°C. The best post-thaw motility and fertilization results, 56.67±5.77% and 80.5±16.01% respectively, were obtained when a glucose based extender was used.

Introduction

Cryopreservation of fish semen can be very useful in hatcheries. It has applications in broodstock management, selective breeding and gene conservation. Stocks can be protected from total elimination due to sudden disease outbreaks and natural disasters. Cryopreservation of fish semen could reduce the number of males needed in a hatchery, minimize handling stress through less frequent stripping and facilitate genetic research (Akcay et al., 2002). Most experimental work in this field has focused on finding the optimal saline solutions, cryoprotective agents, thawing solutions and freezing and thawing rates for salmonids. Although differences in diluents, cryoprotectants and freezing techniques cause a wide variation of fertilization results, the most reliable indicator of successful semen cryopreservation is the fertilization rate.

* Corresponding author. E-mail: ntekin@veterinary.ankara.edu.tr

However, there are few and varied fertilization results relating to cryopreservation of salmonid species.

The objective of this study was to examine the effects of different extenders containing egg yolk and DMSO as cryoprotectants on the fertility of rainbow trout *(Oncorhynchus mykiss* W. 1792) sperm.

Materials and Methods

Adult fish and semen collection. Adult male and female rainbow trout (2-5 years old) were obtained from the Fish Production Station in Bolu, Turkey. In the pre-spawning period the fish were kept seperately in small ponds under constant environmental conditions and fed a pelleted diet (50% protein). The water temperature ranged 7-12°C during the spawning period. For 48 hours prior to collecting the semen, the fish received no feed. The semen was collected into 50 ml calibrated glass beakers from 25 mature rainbow trout by abdominal massage. Samples contaminated with fecal material or urine were discarded. Eggs were stripped from two mature females by gently massaging the abdomen. The eggs used for the experiments were well-rounded and transparent.

Motility, movement duration, concentration and pH of sperm. Motility was evaluated by placing about 10 µl semen on a glass microscope slide and adding 100 µl activation solution (0.3% NaCl). The samples were examined under a light microscope at x 40 magnification and motility was expressed as the percent of spermatozoa that were motile. Only samples showing >80% motility were used for cryopreservation. The duration of the spermatozoa movement was measured with a sensitive chronometer. The semen concentration was estimated by the hemocytometric method and expressed as spermatozoa x 10^9 per ml. pH was measured with indicator papers.

Extenders. Semen showing >80% motility from ten males was pooled in equal amounts and kept at 4°C prior to dilution. The pooled semen was diluted at a ratio of 1:3 (semen:extender) with one of three extenders. Extender I contained 125 mM sucrose, 6.5 mM glutathione, 100 mM $KHCO_3$, 10% fresh hen egg yolk and 10% DMSO as described by Mounib (1978). Extender II was prepared as described by Lahnsteiner et al. (1997) and contained 103 mM NaCl, 40 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM Hepes, 1.5% BSA (w/v), 7% egg yolk, 0.5% sucrose (w/v) and 10% DMSO. Extender III was glucose-based and contained 300 mM glucose, 10% egg yolk and 10% DMSO.

Cryopreservation and thawing of semen. Following dilution, the semen samples were drawn into 0.5 ml plastic straws (IMV, France) and sealed with polyvinile alcohol (PVA). The diluted samples were equilibrated about 2-5 min at 4°C. Then the labelled straws were laid horizontally on a tray above liquid nitrogen in a styrofoam box. The straws were frozen in the vapor of the liquid nitrogen, 3 cm above the surface of the liquid, for 10 min and then plunged into the liquid nitrogen. The straws were transferred to a liquid nitrogen container and stored for several days. The frozen straws were thawed in a water bath at 30°C for 10 sec and activated using 0.3% NaCl. The motility and duration of the motility were tested and recorded. The thawed semen was used immediately for fertilization.

Fertilization. The thawed straws were cut open and the semen was poured onto the eggs. The semen and eggs were gently mixed about 10 sec and the fertilization solution (3 g urea, 4 g NaCl and 1 l water) was added. About ten minutes after fertilization, the eggs were rinsed in hatchery water and incubated in a vertical egg incubator. The success of the experiment was determined as the percent of eyed embryos about 25 days after fertilization. The dry fertilization tests were done in triplicate in sterile petri dishes at 4°C. Fertilization tests were performed using six 0.5 ml straws of cryopreserved semen to fertilize each 30 g (about 600) eggs. The same semen and egg pools were used for related experiments to exclude the influence of variability in gamete quality. Fertilization with untreated (control) semen was performed in a similar way and at a similar sperm:egg ratio.

Statistical analysis. Significant differences among means were detected using one-way analysis of variance (ANOVA) by MINTAB 13 software.

Results

Highest and mean values of spermatological properties before treatment are summarized in Table 1. The effects of the three extenders on the sperm are summarized in Table 2.

Discussion

The mean semen volume in this study was similar to results reported by Buyukhatıpoglu and Holtz (1984) and Geffen and Evans (2000) for rainbow trout. On the other hand, results reported by McNiven et al. (1993) and Lahnsteiner et al. (1993) differed from our findings. The differences may be due to feeding conditions and regimes, water quality or other environmental factors. It is generally known that cryopreserved semen motility is lower than motility of fresh spermatozoa. In our study, the mean motility of the fresh semen (Table 1) was similar to that found by Schmidt-Baulain and Holtz (1989) and Levanduski and Cloud (1988) while post-thaw motility (Table 2) agreed with the findings of Moccia and Munkittrick (1987) and Cabrita et al. (2001). The mean concentration of spermatozoa in our study agrees with Munkittrick and Moccia (1987) and McNiven et al. (1993) but not with Buyukhatipoglu and Holtz (1984) who reported a concentration of 12.71±6.37 x 10^9/ml. The difference may be due to dilution ratio, age or breeding season. The mean pH in our study was generally confirmed by Piironen (1985) and Munkittrick and Moccia (1987).

The poorest post-thaw motility and fertilization rates were obtained with extender I while the highest were obtained with extender III. The differences may be explained by the cryoprotective effects of sucrose and glucose on spermatozoa. Our extender III fertilization rate was similar to the rate (90%) found by Ohta el al. (1995) for Masu salmon semen cryopreserved in a glucose-DMSO 10% based extender.

Another factor affecting the fertilization rates was the use of 0.5 ml straws. The fertilization rate in our study agrees with Lahnsteiner (2000) who received the best fertilization results when semen was frozen in straws with a volume of ≤1.2 ml. Similarly,

Table 1. Volume, motility, duration of motility, concentration, number and pH of spermatozoa in fresh rainbow trout (*Oncorhynchus mykiss* W. 1792) semen (n = 25).

	Volume (ml)	*Motility (%)*	*Movement duration (sec)*	*Concentration (10^9/ml)*	*No. (10^9)*	*pH*
Mean value (±sd)	19.72±16.05	76.68±12.88	98.6±50.6	7.2±4.21	136.80±135.06	7.18±0.28
Highest value	62	90	202	19.1	612	7.5

Table 2. Effects of extenders on parameters in cryopreserved rainbow trout *(Oncorhynchus mykiss* W. 1792) semen (n = 3).

	*Post-thaw motility (%)**	*Post-thaw movement (sec)**	*Fertilization rate (%)***	*Hatching rate (%)**
Extender I (mean±SD)	26.67±11.55[a]	32.33±13.79[a]	25.08±14.52[a]	98.21±0.03[a]
Extender I (highest)	40	48	37.6	98.24
Extender II (mean±SD)	40.00±10.00[ab]	60.33±86.00[ab]	12.97±9.51[a]	98.33±2.88[a]
Extender II (highest)	50	67	21.3	100
Extender III (mean±SD)	56.67±5.77[b]	84.33±20.03[b]	80.5±16.01[b]	96.70±2.69[a]
Extender III (highest)	60	107	94.6	99.4
Control (mean±SD)			92.47±1.74[b]	97.67±2.51[a]
Control (highest)			94.4	100

Means within a column marked with different letters are significantly different.
* significant at $p<0.05$
** significant at $p<0.01$

Wheeler and Thorgard (1991) obtained only intermediate fertilization rates (about 50%) when rainbow trout semen was cryopreserved in 4.5 ml straws. In the present study, an average ratio of 3×10^6 motile sperm to one egg was used, which may be another reason for the low fertilization results obtained with extenders I and II. According to Lahnsteiner et al. (1996), the sperm concentration should be 2-2.5 x 10^9 cells per ml of diluent. At higher cell concentrations, the post-thaw fertility of semen decreases, probably due to cell damage caused by limited intracellular space and cell compression. In addition to these factors, another reason for the low fertilization obtained with extenders I and II might be that the thawed semen was exposed to the air, even though for less than 30 sec, possibly causing cell injuries from, perhaps, swelling.

The present study demonstrates that rainbow trout spermatozoa in a glucose-based extender containing 10% DMSO can be succesfully cryopreserved and achieve a fertilization rate similar to that of fresh spermatozoa. Further studies are needed to discover the optimum sperm:egg ratio to increase the efficiency of the cryopreserved sperm and fertilize the larger egg batches used in aquaculture.

References

Akcay E., Bozkurt Y. and S. Kayam, 2002. Cryopreservation of mirror carp (*Cyprinus carpio* L. 1758) semen: with emphasis on post-thaw motility. pp. 5. In: *1st Int. Cong. Aquacult. - Fish. Technol. Environ. Management.* June 8-10, 2002. ECEP, Book of Abstracts, Athens.

Buyukhatipoglu S. and W. Holtz, 1984. Sperm output in rainbow trout (*Salmo gaird-*

neri) - effect of age, timing and frequency of stripping and presence of females. *Aquaculture*, 37:63-71.

Cabrita E., Robles V., Alvarez R. and M.P. Herraez, 2001. Cryopreservation of rainbow trout sperm in large volume straws: application to large scale fertilization. *Aquaculture*, 201:301-314.

Geffen A.J. and J. Evans, 2000. Sperm traits and fertilization success of male and sex reversed female rainbow trout. *Aquaculture,* 182:61-72.

Lahnsteiner F., 2000. Semen cryopreservation in the Salmonidae and in the northern pike. *Aquacult. Res*., 31:245-258.

Lahnsteiner F., Patzner R.A. and T. Weismann, 1993. Energy resources of spermatozoa of the rainbow trout, *Oncorhynchus mykiss*. (Pisces, Teleostei). *Reprod. Nutr. Dev*., 33:349-360.

Lahnsteiner F., Berger B., Weismann T. and R.A. Patzner, 1996. Changes in morphology, physiology, metabolism, and fertilization capacity of rainbow trout semen following cryopreservation. *Prog. Fish-Cult.,* 58:149-159.

Lahnsteiner F., Weismann T. and R.A. Patzner, 1997. Methanol as cryoprotectant and the suitability of 1.2 ml and 5 ml straws for cryopreservation of semen from salmonid fishes. *Aquacult. Res*., 28:471-479.

Levanduski M.J. and J.G. Cloud, 1988. Rainbow trout (*Salmo gairdneri*) semen: effect of non-motile sperm on fertility. *Aquaculture*, 75:171-179.

McNiven M.A., Gallant R.K. and G.F. Richardson, 1993. Dimethyl-acetamide as a cryoprotectant for rainbow trout spermatozoa. *Theriogenology*, 40:943-948.

Moccia R.D. and K.R. Munkittrick, 1987. Relationship between the fertilization of rainbow trout (*Salmo gairdneri*) eggs and the motility of spermatozoa. *Theriogenology*, 27:679-688.

Mounib M.S., 1978. Cryogenic preservation of fish and mammalian spermatozoa. *J. Reprod. Fertil.*, 53:13-18.

Munkittrick K.R. and R.D. Moccia, 1987. Seasonal changes in the quality of rainbow trout (*Salmo gairdneri*) semen: effect of a delay in stripping on spermatocrit, motility, volume and seminal plasma contituents. *Aquaculture*, 64:147-156.

Ohta H., Shimma H. and K. Hirose, 1995. Relationship between fertility and motility of cryopreserved spermatozoa of amogo salmmon *Oncorhynchus masou ishikawae. Fish Sci*., 61:886-887.

Piironen J., 1985. Variation in the properties of milt from the Finnish landlocked salmon (*Salmo salar* M. Sebago Girard) during a spawning season. *Aquaculture*, 48:337-350.

Schmidt-Baulain R. and W. Holtz, 1989. Deep-freezing of rainbow trout (*Salmo gairdneri*) sperm at varying intervals after collection. *Theriogenology,* 32(3):439-443.

Wheeler P.A. and G.H. Thorgaard, 1991. Cryopreservation of rainbow trout semen in large straws. *Aquaculture*, 93:95-100.