# Effect of Different Cryoprotectants on Viability of Mirror Carp (*Cyprinus carpio*) Spermatozoa

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**ABSTRACT**

The study investigated effects of different cryoprotectants on post-thaw viability of cryopreserved mirror carp (*Cyprinus carpio*) spermatozoa. Volume, motility, movement duration, concentration, total spermatozoa number and pH were recorded in collected semen. Following determination of spermatological properties, the semen was diluted with an extender containing three different cryoprotectants and frozen in liquid nitrogen vapour (-196°C) in 0.5 ml straws for 10 min. The straws were thawed in a water bath at 30 ºC for 30 seconds. The highest post-thaw motility was determined as mean 35.2±1.45 % in extender containing DMSO. On the other hand, the highest post-thaw movement duration was determined as mean 115.2±2.46 s in extender containing glycerol. In conclusion, Kurokura solution containing 15 % DMSO and glycerol can be successfully applied to the cryopreservation of mirror carp semen.

**Key words:** Mirror carp, *Cyprinus carpio*, semen quality, cryoprotectant, male broodstock.

# Farklı Kriyoprotektantların Aynalı Sazan (*Cyprinus carpio*) Spermatozoalarının Canlılığı Üzerine Etkisi

**ÖZET**

Bu çalışmada, farklı kriyoprotektantların dondurulmuş aynalı sazan (*Cyprinus carpio*) spermatozoalarının çözüm sonu canlılıkları üzerine olan etkisi araştırıldı. Toplanan spermada miktar, motilite, canlılık süresi, yoğunluk, toplam spermatozoa sayısı ve pH belirlendi. Spermatolojik özelliklerin belirlenmesinin ardından sperma üç farklı kriyoprotektant içeren sulandırıcı ile sulandırıldı ve 0.5 ml'lik payetler içerisinde 10 dk. süre ile sıvı nitrojen buharında (-196 °C) donduruldu. Payetler 30 °C su banyosunda 30 saniye süre ile çözdürüldü. Çözüm sonu ortalama en yüksek motilite DMSO içeren sulandırıcıda 35,2±1,45 olarak belirlendi. Çözüm sonu ortalama en yüksek canlılık süresi ise gliserol içeren sulandırıcıda 115,2±2,46 s. olarak belirlendi. Sonuç olarak, % 15 oranında DMSO ve gliserol içeren Kurokura solüsyonu aynalı sazan spermasının dondurulmasında başarılı bir şekilde uygulanabilir.

**Anahtar kelimeler:** Aynalı sazan, *Cyprinus carpio*, sperma kalitesi, kriyoprotektant, erkek damızlık stoğu.

## INTRODUCTION

Cryopreservation of semen could reduce the number of males needed in the hatchery, minimize handling stress through less frequent stripping, facilitate genetical researchs and lead to the development of inbred lines.

The practice of cryopreservation includes cooling, freezing and thawing of semen. Performing in this task involves: 1)using of apprropriate cryoprotectant solution to minimize cellular disruption and membrane damage from ice crystal formation 2) lowering of temperature to the temperature of liquid nitrogen (-196 °C) and 3) following of proper methods to prevent damage during thawing (Brown and Brown, 2000). Differences in diluents, cryoprotectants and freezing techniques make it difficult to get variable estimations of the efficiency of various procedures (Lubzens et al., 1997).

Cryopreservation of carp semen has been attempted by many authors (Kurokura et al., 1984, Linhart et al., 1988, Cognie et al., 1989, Zhang and Lio, 1991). Most of these experiments in these field have focussed on finding optimal saline solutions and cryoprotective agents for carp. Generally two types of extenders have been developed for the cryopreservation of fish spermatozoa: seminal plasma resembling media (Kurokura et al., 1984) and simple carbohydrate-based solutions (Stoss and Holtz, 1983, Cognie 1989). Mostly dimethyl sulfoxide (DMSO) is used as the permeating cryoprotective agent for cryopreservation, but other cryoprotectants like dimethyl acetamide (DMA), ethylene glycol, glycerol and DMSO-glycerol mixture are also efficient (Linhart et al., 1988, Drokin et al., 1989, Cognie et al., 1989). Especially using an appropriate cryoprotectant solution prevents cells from cellular disruption and membrane damage during freezing and thawing (Brown and Brown, 2000). However, there is a little data on milt quality in connection with cryopreservation.

The simplest and most reliable indicator of the semen quality is spermatozoa motility. The criterion is commonly used in the selection of milt for insemination and preservation. The spermatozoa motility varies in vigor and duration not only among males but also within an individual male depending on its ripeness. In numerous fish species with external fertilization, duration of spermatozoa motility is very short (Billard and Cosson, 1989). The highest motility of the spermatozoa is observed at the height of the breeding season (Terner, 1986). Studies on most of the fish species reported the duration and motility of semen may show seasonal variation (Benau and Terner, 1980).

Futhermore, spermatozoa motility is an important component of a cryopreservation program in order to prevent poor quality semen samples prior to freezing and to estimate the fertility of the stored semen after thawing. In Cyprinid fishes, motility is often used to estimate semen quality and viability when spermatozoa exposed to cryopreservation; a relationship between motility and fertilizing capacity has been assumed by several authors (Billard and Cosson, 1992; Lahnsteiner et al., 1996). Especially post-thaw motility is one of the important indicators of an successfull cryopreservation.

From this point of view, the main purpose of this study was to investigate the effects of differents cryoprotectants on post-thaw viability of cryopreserved mirror carp (*Cyprinus carpio*) spermatozoa.

## MATERIALS and METHODS

### Adult Fish and Care

Twenty adult males of mirror carp (*Cyprinus carpio*) (2.350± 0.525 kg of body weight and 47.92 ± 3.23 cm total length) were obtained from a local fish farm in Bolu. In the pre-spawning period the parenteral broodfish were kept separately in small ponds and fasted 48 hour prior to semen collection.

### Semen Collection

Semen was collected from anesthesized males (0.1g/l, MS-222) by hand stripping method 12 h after a single injection of 2 mg/kg carp pituitary extract (CPE) (Saad and Billard, 1987) under dorsal fin. Their abdomens and urogenital papillas were dried before stripping. Semen was collected into 50 ml glass tubes. Samples contaminated with faecal matterial or urine were discarded.

### Evaluation of Motility, Movement Duration, Concentration and pH

Spermatozoa motility was evaluated using a light microscope (x400) and was expressed as percentage of motile spermatozoa. NaCl 0.3% as activating solution was used. Only samples showing high motility (>80 %) were used for freezing. Movement duration was estimated using a sensitive chronometer (1/100 s). Motility and movement duration were evaluated according to the following criteria: 1)Mass progressive motility when most of the spermatozoa were still actively swimming with progressive movement 2)Total duration of movement until most spermatozoa stopped swimming. The spermatozoa concentration was estimated using the hemocytometric method and expressed spermatozoa number $x10^9$/ml. pH was measured by indicator papers (Merck 5.5-9.0) (Billard et al., 1989).

### Dilution

Semen samples showing high motility (>80 %) from 7 males were pooled in equal amounts. The pooled semen was diluted at a ratio of 1:3 with extender containing three different cryoprotectants (15% DMSO, 15%DMA and 15% Glycerol). Extender contained 0.75 g NaCl, 0.02 g KCl, 0.02 g $CaCl_2$ and 0.02 g $NaHCO_3$ supplemented to 100 ml with distilled water described by Kurokura et al. (1984). The semen and diluent were kept at 4°C prior to dilution.

### Packaging and Equilibration

The diluted samples were drawn into 0.5 ml plastic straws (IMV, France) and were sealed with polyvinile alcohol (PVA). The samples were equilibrated for 45 min at 4°C.

### Freezing

After equilibration, the straws were placed on a styfoam rack that floated on the surface of liquid nitrogen in a styfoam

box. The straws were frozen in liquid nitrogen vapour 3 cm above of the surface of liquid nitrogen for 10 min. After 10 min the straws were plunged into the liquid nitrogen and stored for one day.

**Thawing**

Frozen straws were thawed by plunging into water at 30°C for 30 s. Thawed semen were acivated using 0.3% NaCl and motility and duration of motility were tested and recorded again.

**Statistical Analyses**

Results are expressed as mean±standart deviation. The results were analysed by variance analysis (ANOVA) and significant differences were detected using Duncan test. All statistical analysis were carried out using SPSS 11 for windows software package.

**RESULTS**

The spermatological properties of the semen are presented in Table 1. Semen volumes were rather variable and ranged from 2.5 to 37 ml and mean volume was 15±13.73 ml. Motility values were rather high and ranged from 70 to 90%. Movement duration was also varied between 315 and 858 s and determined as mean 544.42±199.43 s. Mean spermatozoa concentration and total spermatozoa number was determined as 19.614±4328.27 $x10^9$/ml and 267.4±214.47 $x10^9$ respectively. Semen pH was determined as mean 7.85±0.24.

Effects of cryoprotectants on post-thaw motility and movement duration are presented in Table 2. The highest post-thaw motility was determined as mean 35.2±1.45% with DMSO. In addition, the highest movement duration was found as mean 115.2±2.46 s with glycerol. Differences between the means of movement durations were significant ($p<0.05$).

**DISCUSSION**

Mean sperm volume of mirror carp was similar to results reported by Akçay et al (2004) but found higher than results of the Bozkurt and Seçer (2005). The differences may be due to the feeding conditions and regime, environmental factors or spawning time.

Similarly, the mean spermatozoa motility was similar to the results of Akçay et al (2002, 2004) but found higher from those of Bozkurt and Seçer (2005) for mirror carp. Duration of spermatozoa movement was found similar to the results of Akçay *et al.* (2004) but found higher than Bozkurt and Seçer (2005). Spermatozoa motility varies in vigor and duration not only among males but also within an individual male depending on ripeness (Akçay *et al.* 2002). Most studies on fish species have shown that the duration and motility of semen may vary seasonally (Benau and Terner, 1980; Akçay *et al.* 2004). Spermatozoa concentration was found rather similar to the results of Akçay et al. (2004) but not those reported by Emri et al. (1998). The difference may be due to differences in feeding conditions, spawning time or dilution ratio. The mean pH generally confirmed by Saad et al. (1988) and Lubzens et al. (1997).

Results regarding to the post-thaw motility was determined as mean 35.2±1.45% and lower than that of Akçay et al. 2002. It was observed that a decrease in movement duration occurred following cryopreservation. The longest movement duration were achieved (115.2±2.46 s) when using glycerol as cryoprotectant. Similarly Akçay et al. (2002) obtained the highest post-thaw movement duration about as 180 s when glycerol was used.

Semen quality and freezing protocols are usually evaluated by analyzing different parameters, such as motility or cell viability during cryopreservation. Especially, post-thaw motility is one of the most important indicators of the success of a freezing protocol. It can be clearly seen that mirror carp spermatozoa motility was affected during cryopreservation in the present study. Furthermore, movement duration was also affected. Similar results for the motility parameters of frozen thawed spermatozoa were reported in fish in some experiments (Kurokura et al. 1984, Linhart et al. 1988).

During freezing and thawing, spermatozoa are subjected to severe physical shocks resulting injuries of cell membranes. This affects spermatozoal fertilization ability closely dependent on both cell membrane and genome integrity. Cryoprotectants stabilize proteins in unfrozen, aqueous solution, but alternatively can also induce protein denaturation at high temperatures and cause cryoprotectant toxicity in cellular systems (Chao, 1991). During freezing, high concentrations of cryoprotectants, which may be lethal to unfrozen cells, can prevent ice formation. The addition of cryoprotectants to the semen greatly extends the tolerance of spermatozoa to freezing at slower rates and it is agreed that the optimal cooling rate depends on the nature and concentration of the cryoprotectant used (Chao, 1991). It has also been reported that cryoprotectants are most effective when they can rapidly penetrate the cell during freezing, and delay intracellular freezing and minimize the solution effect (Simione, 1998).

Some authors recommend having an equilibration time after predilution, allowing internal cryoprotectants to penetrate the spermatozoa before freezing (Steyn et al., 1989). However, such a delay was not observed to improve cryopreservation success in trout (Lahnsteiner et al., 1996, Stoss and Holtz 1983). In the present study, 45 minutes equilibration times were applied following dilution of semen and affected positively the viability of spermatozoa.

In conclusion, the present study indicates that mirror carp semen can be successfully cryopreserved with Kurokura solution by adding 15% DMSO and glycerol. The information on sperm physiology obtained from the present study can lead to more efficient gamete management and increased fry yields and aid suitability of sperm for frozen.
On the other hand, additional studies are necessary to evaluate the viability, survival and development of larvae produced from frozen semen.

Table 1. Spermatological properties of mirror carp (*Cyprinus carpio*) (n=20)

| | Semen volume (ml) | Spermatozoa motility (%) | Movement duration (s) | Spermatozoa density ($x10^9$/ml) | Total no. spermatozoa ($x10^9$) | Semen pH |
|---|---|---|---|---|---|---|
| Means±SD | 15±13,73 | 86.42±8.52 | 544.42±199.43 | 19.614±4328.27 | 267.4±214.47 | 7.85±0.24 |
| Range | 2.5-37 | 70-90 | 315-858 | 15.375-24.625 | 18.3-304.6 | 7.5-8.0 |

Table 2. Effect of cryoprotectants on mean (±SD) post-thaw motility and movement duration and pH (n=3)

| | Cryoprotectants | Motility (%) | Movement duration (s) | pH |
|---|---|---|---|---|
| Extender | 15% DMSO | 35.2±1.45 [a] | 72.3±2.30[c] | 7.5.2±3.6 [a] |
| | 15% DMA | 10.4±2.36 [a] | 10.5±3.20[b] | 7.5.1±1.4 [a] |
| | 15% Glycerol | 30.5±1.24 [a] | 115.2±2.46[a] | 7.5.4±2.7 [a] |

Different superscripts in a column indicate significant differences at $p<0.05$.

**REFERENCES**


Akçay 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. Athens (abstract).

Akçay, E., Bozkurt, Y., Seçer, S., and Tekin, N. 2004. Cryopreservation of mirror carp semen. Turk. J. Vet. Anim. Sci. 28: 837-843.

Benau D. and C. Terner, 1980. Initiation, prolongation and reactivation of the motility of salmonid spermatozoa. *Gamete Res.*, 3: 247-257.

Billard, R., Cosson M. R. 1989. Measurement of sperm motility in trout and carp. In: Aquaculture (A Biotechnology in Progress), European Aquaculture Society, Breden, Belgium, pp: 499-503.

Billard, R. and Cosson, M. R. 1992. Some problems related to the assesment of sperm motility in freshwater fish. Journal of Experimental Zoology. 261:122-131.

Bozkurt, Y. and Seçer, S. 2005. Effect of short-term preservatıon of mirror carp (*Cyprinus carpio)* semen on motılıty, fertilization, and hatching rates. The Israeli Journal of Aquaculture – Bamidgeh 57(3) 207-212.

Brown, G. G. and Brown, L. D. 2000. Cryopreservation of sperm of stripped bass and white bass. In: Cryopreservation in Aquatic Species. Tiersch, T.R. and Mazik, P.M. Editors. World Aquaculture Society, Baton Rouge, Louisiana, pp. 130-137.

Chao, N. H. 1991. Fish sperm cryopreservation in Taiwan: Technology advancement and extension efforts. Paper on International Symposium on Reproductive Biology in Aquaculture. Department of Aquaculture, Taiwan Fishery Research Institute, Taiwan; p. 31.

Cognie, F., Billard, R., Chao, N. H. 1989. Freezing of the milt of the common carp, Cyprinus carpio. Journal of Applied Ichtyology. 5 (4):165-176.

Drokin, S., Kopeika, E. F. and Grishchenko, V.I. 1989. Differences in the resistance to cryopreservation and specifics of lipid compositions of spermatozoa of marine and freshwater fish species. Doklady Biochemistry. 304 (1-6) 65-68.

Emri, M., Marian, T., Tron, L., Balkay, L. and Krasznai, Z. 1998. Temperature adaptation changes ion concentrations in spermatozoa and seminal plasma of common carp wihout affecting sperm motility. Aquaculture, 167: 85-94.

Kurokura, H., Hirano, R., Tomita, M. and Iwahashi, M. 1984. Cryopreservation of carp sperm. Aquaculture. 37:267-273.

Lahnsteiner, F., Berger, B., Weismann, T. and Patzner, R.A. 1996. Motility of spermatozoa of Alburnus alburnus (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism Fish Physiology and Biochemistry 15(2): 167-179.

Linhart, O., Liehman, P. and Rab, P. 1988. The first results in freezing of carp semen. Bulletin VURH Vodnany. 24(2): 3-13.

Lubzens, E., Daube, N., Pekarsky, I., Magnus, Y., Cohen, A., Yusefovich, F., Feigin, P. 1997. Carp (*Cyprinus carpio* L.) spermatozoa cryobanks-strategies in research and application. Aquaculture. 155:13-30.

Saad, A. and Billard, R. 1987. Spermatozoa production and volue of semen collected after hormonal stimulation in the carp. Aquaculture. 65:67-77.

Saad, A., Billard, R., Theron, M. C. and Hollebecq, M.G. 1988. Short-term preservation of carp (*Cyprinus carpio*) semen. Aquaculture. 71:133-150.

Simione FP. 1998. Cryopreservation manual. New York: Nalge Nunc International Corp.; p. 8.

Steyn, G. J. , Van Vuren, J. and Grobler, E. 1989. A new sperm diluent for the artificial insemınatıon of rainbow trout. Aquaculture, 83 : 367 - 374

Stoss, J. and Holtz, W. 1983. Cryopreservation of rainbow trout sperm. IV. The effect of DMSO concentration and equilibration time on sperm survival, sucrose and KCl as extender components and the osmolality of the thawing solution. Aquaculture. 32:321-330.

Terner, C. 1986. Evaluation of salmonid sperm motility for cryopreservation. The Progressive Fish Culturist 48: 230-232.

Zhang, X. and Lio, Y. 1991. Study of cryopreservation of fish spermatozoa. Acta Scientiarum Naturalium Universitatis Normalis Hunanensis 14(3): 255-259.