ARTICLE DOI: 10.5504/BBEQ.2011.0018 B&E

EFFECT OF DIFFERENT THAWING RATES ON MOTILITY AND FERTILIZING CAPACITY OF CRYOPRESERVED GRASS CARP (*CTENOPHARYNGODON IDELLA*) SPERM

Ilker Yavas[1], Yusuf Bozkurt[2]
[1]Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Hatay, Turkey
[2]Mustafa Kemal University, Faculty of Fisheries, Department of Aquaculture, Hatay, Turkey
Correspondence to: Yusuf Bozkurt
E-mail: yfbozkurt@yahoo.com

ABSTRACT

*Cryopreservation of spermatozoa has been well developed in many fish species for resource conservation and aquaculture practices. There are limited data on the effect of cryopreservation on grass carp (*Ctenopharyngodon idella*) spermatozoa. This research was carried out to investigate the effect of different thawing procedures on motility and fertilizing capacity of frozen/thawed grass carp semen as preliminary data to design future cryopreservation experiments. Semen was collected by abdominal stripping from adult male grass carp. Collected semen was diluted with extender containing 350 mM glucose, 30 mM Tris and 5% glycerole (pH 8.0). Diluted samples were packaged in 0.25 ml straws and left to equilibrate for 30 min at4°C. Following equilibration, the straws were exposed to liquid nitrogen vapour for 10 min and plunged into the liquid nitrogen (-196°C). For thawing, the straws were removed from liquid nitrogen and immersed in 30, 35 and 40°C water for 10, 20 and 30 seconds. In fertilization experiments spermatozoa:egg ratio was 1x10^5:1. The highest mean motility (83.4±2.1) ($p<0.05$) and fertilization rate (85.6±2.8) ($P<0.05$) was determined from semen thawed at 35ºC for 30 sec. The results indicate that thawing rates significantly influenced motility and fertilizing ability of cryopreserved grass carp semen.*

Biotechnol. & Biotechnol. Eq. 2011, **25**(1), 2254-2257

Keywords: Grass carp, cryopreservation, thawing rate, semen, motility, fertilization

Introduction

The grass carp (*Ctenopharyngodon idella*), also known as the white amur, is a vegetarian fish native to the Amur River in Asia. Because this fish feeds on aquatic plants, it can be used as a biological tool to control nuisance aquatic plant growth. Grass carp is indigenous to those rivers in the eastern part of Russia and China that flow into the Pacific Ocean. In some countries, the grass carp is an integral part of fish culture and forms an important source of protein for human consumption (26).

Cryopreservation technique provides many benefits, such as conservation of genetic diversity, selective breeding, hybridization, and stable supply of gametes for hatchery seed production or laboratory experimentation (20, 25). The viability of sperm could be preserved theoretically for 200-32 000 years in liquid nitrogen (-196ºC) (2). For this reason, development of an effective sperm cryopreservation protocol has been recommended to the aquaculture industry as a method to improve breeding programmes.

On the other hand, multiplicity of cryopreservation procedures could affect the consistency of fertilization results. Cryopreservation techniques involve addition of cryoprotectants and freezing-thawing of sperm samples, all of which may result in some damage to the spermatozoa and may decrease egg fertilization rates. Furthermore, the methods of fish sperm cryopreservation reveal several common problems such as considerable variability in spermatozoa motility or a wide range of fertility in spermatozoa survival after thawing (28).

Therefore before cryopreservation of sperm, a thorough evaluation of different extender solutions, cryoprotectants, cooling and thawing rates is essential in order that optimal cryopreservation protocols for various species are developed. While evaluating the efficacy of the cryopreservation procedure, it is essential to estimate post-thaw motility of sperm as a guide for their ability to fertilize the eggs successfully (23).

Although methods for sperm cryopreservation have been reported in many fish species, principally in large, commercial species such as salmonids and marine teleosts (5, 6, 25, 27) few studies have addressed this subject to the grass carp (7, 30). The availability of a reliable method for cryopreservation of grass carp sperm may help to improve the efficiency of reproductive management in hatcheries. For this reason, grass carp sperm cryopreservation requires further examination.

Sperm motility is often used to determine semen quality and viability when spermatozoa are exposed to various experimental conditions (4). In addition a relationship between motility and fertilizing capacity has been assumed by several authors (1). Therefore sperm 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 sperm after thawing.

From this point of view it seems that, post-thaw motility and fertilizing ability are very important for cryopreservation of fish sperm. The main purpose of this study was to investigate the effect of different thawing procedures on motility and fertilizing ability of frozen grass carp spermatozoa.

Materials and Methods

Broodstock management

The broodstock were held in earthen ponds under natural photoperiod regime at State Hydraulic Works (SHW) Fish Production Station, Adana. In these ponds water temperature varied between 23 and 25°C during spawning season. The broodstock was collected from wintering ponds by seining and transported into the hatchery 48 h prior to gamete collection. In the hatchery, male and female broodfish were held separetely in shadowed tanks (V=1000 L) supplied with continuously (2.5 L min^{-1}) well-aerated water of 24ºC. Fish were not fed during the experiment.

Gamete collection

The experiment was carried out with mature 50 male and 10 female grass carp. Spermiation and ovulation was induced with intraperitoneally injected GnRH analogue, Ovopel, which is obtainable as pellets weighing approximately 25 mg (11). Each pellet contains 18-20 µg of [D-Ala6, Pro9] NEt-mGnRH and 8-10 mg of the dopamine D2 receptor antagonist, metoclopramide. Males were injected with 1 pellet Ovopel per kg body weight while females were injected with 1.5 pellet Ovopel per kg body weight that were suspended in 0.65% NaCl solution. Males received a single dose 24 h before the planned stripping while females received a priming dose (1/2 pellet) 24 h prior to stripping and a resolving dose (1 pellet) 12 h later (13).

Before gamete collection the broodfish were anaesthetized in a 5 ppm solution of Quinaldine (Reanal Ltd., Budapest, Hungary). Then, broodfish were removed from the water and their genital apertures were wiped dry. Sperm was stripped from 50 males (4-6 years old; total length 39.2±7.3 cm, weight 950.2±42.6 g) by abdominal massage and collected directly into 20 ml calibrated glass beakers. Special care was taken to avoid contamination with urine, mucus, feces or water. Sperm samples were not pooled and the beakers were covered with parafilm and stored on ice under aerobic conditions. Eggs were collected from 10 females (3-4 years old; total length 47.2±4.8 cm, weight 936.2±72.9 g) that had been stripped by gently massaging the abdomen. Eggs were checked visually and only those with homogenous shape, colour and size were used for the fertilization experiments within 1 h post-collection.

Semen evaluation

Spermatozoa were activated on a microscope slide by adding 1 µL of semen to 50 µL of activation solution: 10 mM Tris, 20 mM NaCl, 2 mM $CaCl_2$, pH 8.5 (12). The motility observations were performed by using a dark field microscope at 40x magnification at 4°C using a cold table and expressed as the percentage of cells with progressive motility. To validate motility measurements, preliminary tests were carried out using three samples. For cryopreservation experiments, samples below 80% motile spermatozoa were discarded.

Duration of sperm motility was determined using a sensitive chronometer (sensitivity: 1/100 s) by recording the time following addition of the activation solution to the sperm samples. Spermatozoa density was determined using a 100 µm deep Thoma haemocytometer (TH-100, Hecht-Assistent, Sondheim, Germany) at 40x magnification with Olympus BX50 phase contrast microscope (Olympus, Japan) and expressed as spermatozoa $x10^9$ ml^{-1} (three replicates). Sperm pH was measured using indicator papers (Merck 5.5-9) before counting.

General procedure for sperm dilution, freezing and thawing

Collected semen showing >80 motility was pooled into equal aliquots according to the required semen volume and sperm density to eliminate effects of individual variability of gamete donors. Semen and extenders were kept at 4°C prior to dilution. Pooled semen was diluted at 1:3 ratio (semen/extender) with extender containing 350 mM glucose, 30 mM Tris and 5% glycerole (pH 8.0). Dilution of semen with extender resulted in sperm concentrations of around (1.0 to 2.5)x 10^9 cells/ml extender that was enough to avoid damage due to sperm compression during freezing and thawing (16).

Within 1 h after sperm collection, the diluted semen samples were drawn into 0.25 ml plastic straws (IMV, France) and were sealed with polyvinyl alcohol (PVA). Then, the semen samples in straws were equilibrated for 30 min at 4°C. Following equilibration, the straws were placed on a styrofoam rack that floated on the surface of liquid nitrogen in a styrofoam box. The straws were frozen in liquid nitrogen vapour 6 cm above the surface of liquid nitrogen (temperature of styroframe surface was about -140ºC) for 10 min. After 10 min the straws were plunged into the liquid nitrogen (-196ºC) and stored for several days.

For thawing, the straws were removed from liquid nitrogen and immersed in 30, 35 and 40ºC water for 10, 20 and 30 seconds. Thawed sperm was activated using 0.3% NaCl and observed under microscope for determination of spermatozoa motility and motility durations.

Fertilization experiments

To determine fertilization rates, pooled eggs from 10 females were used. Fertilization took place in dry Petri dishes and egg samples (2 g) (about 100-200 eggs) were inseminated with either fresh or frozen-thawed sperm immediately following thawing. In fertilization experiments spermatozoa:egg ratio was $1x10^5$:1 that was found to be optimal for successful fertilization by Magyary et al. (21). Eggs were inseminated according to dry fertilization technique using a solution of 3 g urea and 4 g NaCl in 1 l distilled water. The sperm and eggs were slightly stirred for 30 min, washed with hatchery water (24ºC, 9 mg l^{-1} O_2) and then gently transferred to labelled Zuger glass incubators with running water (24°C) where they

were kept until hatching (3-4 d). Fertilization experiments were performed in 3 replicates. Living and dead eggs were counted in each incubator during incubation and dead eggs were removed. When the fertilized eggs developed to embryos at the gastrula stage, the fertilization rates (number of gastrula stage embryos/number of total eggs) were calculated.

Statistical analysis

Results are presented as means ± SE. 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 and Discussion

The mean semen volume, spermatoza motility, motility duration, spermatozoa density and pH values of the 50 collected fresh milt samples were as 7.6±2.8 ml, 91.6±6.4%, 72.3±2.46 s, 16.8 x 10^9 ml^{-1} and 7.2, respectively.

TABLE 1

Effect of diffetent thawing rates on motility of spermatozoa and fertilization of eggs in grass carp

Thawing Temperature	Thawing Period	Motility (%)	Fertilization Rates (%)
30ºC	10 sec	67.5±3.4^d	61.8±2.7^d
30ºC	20 sec	72.3±2.6^c	64.2±3.6^d
30ºC	30 sec	73.6±4.7^c	67.3±4.2^d
35ºC	10 sec	79.6±5.9^b	79.5±2.4^c
35ºC	20 sec	81.6±1.7^b	81.9±3.7^b
35ºC	30 sec	83.4±2.1^b	85.6±2.8^b
40ºC	10 sec	73.5±2.4^c	72.6±1.1^c
40ºC	20 sec	64.4±3.4^d	64.5±2.3^d
40ºC	30 sec	75.1±1.1^c	76.9±1.3^c
Control	-	91.6±6.4^a	94.4±4.3^a

Mean values superscripted by the same letter are not significantly different (P>0.05)

Effect of different thawing temperatures and periods on motility and fertilization capacity of cryopreserved grass carp semen is shown in **Table 1**. Mean motility of fresh (control) grass carp semen was 91.6±6.4%. On the other hand, mean fertilization rate of fresh (control) grass carp semen was determined as 94.4±4.3%. Motility and fertilization rates of cryopreserved grass carp semen was determined statistically different ($p<0.05$) between experimental groups. Microscopical view of fertilized grass carp eggs is shown in **Fig. 1**.

Motility is an important characteristic to estimate quality of fresh as well as cryopreserved sperm (15). In the current study, the freezing-thawing process influenced significantly the spermatozoa motility. Similarly, there were significant differences in fertilization rates obtained with frozen semen that were thawed at different periods and temperatures. Similar results for the motility parameters of frozen-thawed spermatozoa were reported in some experiments (14, 15, 20). However, different results were determined in cryopreserved turbot sperm (9), where post-thaw motility was significantly lower than that of fresh sperm.

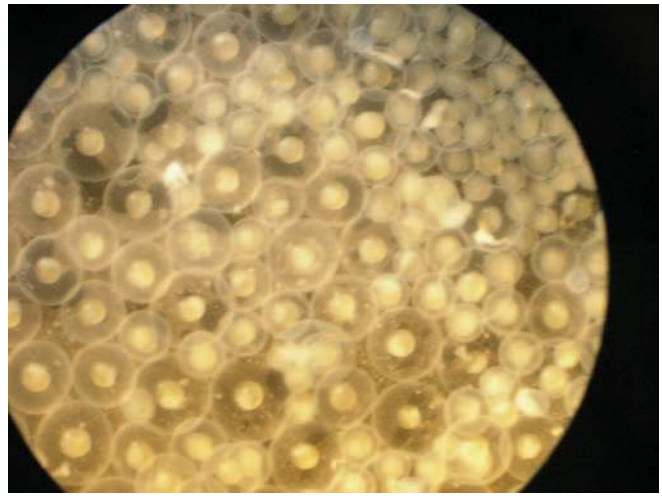

Fig. 1. Microscopical view of grass carp eggs 3-4 h following fertilization

Information regarding to the minimal number of spermatozoa:egg ratio in artificial insemination of the grass carp is not available. The fertilization capacity of eggs is the most important parameter to be evaluated, but using of excess spermatozoa for fertilization masks obviously the quality of frozen spermatozoa. In the present study, fertilization rates were evaluated at a fixed ratio of $1x10^5$:1 spermatozoa:egg according to Magyary et al. (21). It is reported that the concentration of post-thaw sperm used to achieve optimal fertilization success was approximately 100 times higher than that for fresh semen in Cyprinidae (20). Using our protocol with 1:100 000 of egg:spermatozoa ratio, higher fertilization rates were determined regardless of whether frozen or fresh semen was used. On the other hand, Lahnsteiner et al. (18) reported that in cyprinid sperm, the post-thaw fertilization rates obtained with sperm to egg ratios of $1.3\text{-}2.6\times10^6$:1 did not reach that of the fresh sperm. Similar results have also been reported in turbot (24) and flounder (31). These may be due to used extenders, cryoprotectants, equilibration periods, egg quality, or suboptimal protocols applied in sperm cryopreservation procedure. In the present study, a high positive interaction was observed between post-thaw spermatozoa motility and fertilization. This was consistent to results obtained in common carp (19) African Catfish (22) and turbot (10).

Glycerol is one of the most common internal permeable cryoprotectant. So, another important reason for the success of higher fertilization results can be linked to the adequate penetration of glycerol through the cell membranes. This indicates that glycerol has a certain protective effect against freezing damage as a membrane stabilizer. In the present study, diluted samples were equilibrated for 30 min at 4°C. Some authors recommended having an equilibration time after dilution, which would allow cryoprotectants to penetrate spermatozoa before cryopreservation (15). However,

other authors reported that equilibration did not improve cryopreservation success in *Cyprinus carpio* (19, 20).

Thawing rate is critical in preserving viability of the spermatozoa. There is a lack of available data in regards to thawing conditions. Generally, thawing rates should be high to avoid recrystallization (16). Although, Lahnsteiner et al. (17) reported that optimal thawing temperature was 25ºC for the cyprinid spermatozoa, successful post-thaw motility and fertilization results were determined in the present study when frozen semen was thawed at 35ºC and 40ºC. According to the results of the present study it is shown that higher temperatures are necessary to recover membrane stability or metabolism of spermatozoa. Also it appears that either recrystallization and ice crystal formation during thawing were reduced or avoided by this thawing procedure, or enzymatic activities were best reactivated (16). Although thawing from -196ºC to 4ºC is generally considered as a critical phase because of potential recrystallization, the process was similar for all species. Higher temperatures between 30ºC and 40ºC were also used to thaw cyprinid semen in several studies (3, 8, 21).

It is important to keep in mind that, when sperm was tested for fertility, another source of variation is the egg quality. The difficulty in controlling egg quality during fertilization experiments is one of the reasons why sperm quality is mostly evaluated in terms of the sperm motility rate and the percentage of live sperm (29). Since the broodfish used in the present study was well adapted to captive conditions, and the protocol for induction of spawning was well established for this species, the egg quality of all the 10 females was good and allowed test of the fertilization ability of frozen sperm.

Conclusions

The cryopreservation protocol that was developed in the present study was found rather effective and grass carp sperm could be successfully cryopreserved. On the other hand, further studies are needed to evaluate viability, survival, and development of larvae that are produced from cryopreserved grass carp sperm.

Acknowledgements

The authors are also grateful to the staff of SHW Fish Production Station, Adana, Turkey.

REFERENCES

1. **Akcay E., Bozkurt Y., Seçer S., Tekin N.** (2004) Turkish Journal of Veterinary and Animal Sciences, **28**(5), 837-843.
2. **Ashwood-Smith M.J.** (1980) In: Low-temperature preservation in medicine and biology (*M.J. Ashwood-Smith* and J. Farrant, Eds.), Pitman Medicals, London, 19-45.
3. **Babiak I., Glogowsky J., Brzuska E., Szumiec J., Adamec J.** (1995) Aquaculture Research, **28**, 567-571.
4. **Bozkurt Y. and Secer S.** (2005) The Israeli Journal of Aquaculture-Bamidgeh, **57**(3), 207-212.
5. **Cabrita E., Anel L., Hernaez M.P.** (2001) Theriogenology, **56**, 623-635.
6. **Chao N.H. and Liao I.C.** (2001) Aquaculture, **197**, 161-189.
7. **Chen S.L., Liu X.T., Lu D.C., Zhang L.Z., Fu C.J., Fang J.P.** (1992) Acta Zool. Sinica, **38**, 413-424.
8. **Cognie F., Billard R., Chao N.H.** (1989) J. Appl. Ichthyol., **5**, 165-176.
9. **Dreanno C., Suquet M., Quemener L., Cosson J., Fierville F., Normant Y., Billard R.** (1997) Theriogenology, **48**(4), 589-603.
10. **Dreanno C., Cosson J., Sequin F., Dorange G., Billard R.** (1999) Molecular Reproduction and Development, **53**(2), 230-243.
11. **Horvath L., Szabo T., Burke J.** (1997) Pol. Arch. Hydrobiol., **44**, 221-226.
12. **Jahnichen H., Wamecke D., Trolsch E., Kohlmann K., Bergler H., Pluta H.J.** (1999) Journal of Applied Technology, **15**(4-5), 204-206.
13. **Kayim M., Bozkurt Y., Ogretmen F.** (2010) Journal of Animal and Veterinary Advances, **20**(9), 2589-2592.
14. **Kurokura H., Hirano R., Tomita M., Iwahashi M.** (1984) Aquaculture, **37**, 267-273.
15. **Lahnsteiner F., Berger B., Weismann T., Patzner R.A.** (1996) Fish Physiol. Biochem., **15**, 167-179.
16. **Lahnsteiner F.** (2000) Aquaculture Research, **31**, 245-258.
17. **Lahnsteiner F., Berger B., Horvarth A., Urbanyi B., Weismann T.** (2000) Theriogenology, **54**, 1477-1498.
18. **Lahnsteiner F., Berger B., Weismann T.** (2003) Theriogenology, **60**, 829-841.
19. **Linhart O., Rodina M., Cosson J.** (2000) Cryobiology, **41**, 241-250.
20. **Lubzens E., Daube N., Pekarsky I., Magnus Y., Cohen A., Yusefovich F., Feigin P.** (1997) Aquaculture, **155**, 13-30.
21. **Magyary I., Urbanyi B., Horvarth L.** (1996) Journal of Applied Ichthyology, **12**, 117-119.
22. **Rurangwa E., Volckaert F.A.M., Huyskens G., Kime D.E., Ollevier F.** (2001) Theriogenology, **55**, 751-769.
23. **Stoss J. and Holtz W.** (1983) Aquaculture, **32**, 321-330.
24. **Suquet M., Dreanno C., Petton B., Norman Y., Omnes M.H., Billard R.** (1998) Aquatic Living Resources, **11**, 45-48.
25. **Suquet M., Dreanno C., Fauvel C., Cosson I., Billard R.** (2000) Aquaculture Research, **31**, 231-243.
26. **Sutton D.L. and Vandiver V.V.** (2006) University of Florida Bulletin 867, Department of Fisheries and Aquacultural Sciences, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences.
27. **Tekin N., Secer S., Akcay E., Bozkurt Y.** (2003) The Israeli Journal of Aquaculture-Bamidgeh, **55**(3), 208-212.
28. **Tekin N., Secer S., Akcay E., Bozkurt Y., Kayam S.** (2007) Journal of Applied Ichthyology, **23**(1), 60-63.
29. **Viveiros A.T.M and Godinho H.P.** (2009) Fish Physiology and Biochemistry, **35**, 137-150.
30. **Withler F.C.** (1981) Aquaculture, **26**, 395-398.
31. **Zhang Y.Z., Zhang S.C., Liu X.Z., Xu Y.Y., Wang C.L., Sawant M.S., Li J., Chen S.L.** (2003) Theriogenology, **60**, 989-996.