ISSN: 2347-5129
IJFAS 2014; 1(3): 22-25

www.fisheriesjournal.com
Received: 06-10-2013
Accepted: 15-11-2013

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Role of cryopreserved fish spermatozoa as a biotechnological tool in enhancing fish production

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ABSTRACT
Demand for quality fish seeds is prevailing worldwide for cultivable species. Preservation of quality spermatozoa and use of them for artificial fertilization of eggs are advancement in the selective breeding of fishes. The advantages of cryopreservation over the short term preservation were studied with *Cyprinus carpio* spermatozoa. Spermatological parameters (motility duration, motility score and percentage of live cells) were indices for comparison. The results revealed that cryopreservation offered advantages like long term viability of the sperm cells in addition to maintenance of motility. There was a negative correlation between length of storage period and motility duration which was upto 47% in the short term preserved spermatozoa and 17% in the cryopreserved spermatozoa. The cryopreserved spermatozoa had their performance not much reduced, that might be due to the arresting of metabolic activity of the spermatozoa. Spermatological parameters were in favour of cryopreserved spermatozoa indicating the advantages of the cryopreservation method.

Keywords: Cryopreservation, Short term preservation, *Cyprinus carpio*, Spermatozoa, Motility duration

1. Introduction
World aquaculture production shows an increasing trend with an increment of 2.6 million tonnes during 2011 from that of the production reported for 2010 [1]. According to FAO, nearly 600 aquatic species are cultured worldwide[1]. Carps accounted for 40.5 percent of production in aquaculture. Selective breeding and hybridization are considered useful in enhancing the quality of seeds and therefore many attempts have been made in aquatic animals for the production of better seeds for stocking. Knowledge on gametes and application of artificial fertilization has gained attention when there was difficulty in the natural breeding of the fishes such as *Ctenopharyngodon idella* and *Hypophthalmichthys molitrix*. In order to overcome the troubles in breeding and for successful hybridization of fishes, *in vitro* fertilization of eggs with the spermatozoa artificially collected from the male donors is considered useful. Preservation of spermatozoa and use in the *in vitro* fertilization attempts has become necessary in the event of delayed maturation of the females and non-synchronous maturation of both the sexes. Spermatozoa collected from the quality male fishes could be used even after 2 to 3 days by keeping them in 4 ^{0}C. This has led to the concept of short term preservation at 4 ^{0}C [2]. In preserving spermatozoa by short term preservation, spermatozoa are stored under refrigerated condition maintaining the viability for the maximum of 3 weeks [3].
Better survival rates were observed in rainbow trout sperm until day 16 when kept undisturbed [4]. Thus it is clear that the maintenance at low temperature regime of 2 to 6 ^{0}C might be useful for the milt of fishes if they have to be maintained for a short term of 3 to 6 days. In carp semen preserved at 4 ^{0}C the percentages of motile spermatozoa and the fertilizing capacity were high for 2 days and then rapidly decreased to zero after 6-8 days [5].
The spermatozoa of *L. fimbriatus*, when stored at 4 ^{0}C and also at room temperature retained motility up to 84 and 24 h, respectively [6]. On the same line, in undiluted *Schizothoraichthys progastus* semen, 50% of sperm remain motile up to 3 days of storage [7].
An advanced biotechnological approach in the preservation of spermatozoa is cryopreservation wherein spermatozoa are stored by immersing in LN_2 (Liquid nitrogen) for long term [8]. This has been very successful in the long term storage of animal spermatozoa.

There are many successful cryopreservation attempts in fish spermatozoa and artificial fertilization also [9-15]. In all these studies, spermatological parameters are considered as indices for fixing the quality of the spermatozoa [16] and motility is the commonly used parameter to evaluate the sperm quality [17, 18]. *Cyprinus carpio* was one of the principle cultured species and its production during the year 2005 was 2.6 million tonneswhich rose to 3.4 million tonnes in 2010 [1]. Being an omnivore and more tolerant to different water quality, it has been considered as a successful cultivable species in freshwater aquaculture worldwide. Evaluation of preservation methods with the spermatozoa of *C. carpio* is believed to help in the perfection of the technology for field level adoption for many fish species.

The present study was conducted for comparing the viability of *C. carpio* spermatozoa preserved under short term preservation with that of the spermatozoa preserved under cryopreservation in order to find out the advantages of the method that can be effectively followed.

2. Materials and Methods

Adult male brooders of *C. carpio* (ABW of 218±34 g) were maintained in ponds. Matured brooders with oozing milt for a gentle massage in the abdomen were selected. The abdominal region was wiped and cleaned with tissue paper to remove water, mucus and urine [19]. The oozing milt for the second massage onwards was collected in 1.5 ml vials. Care was taken to prevent contamination of milt with urine and blood. The vials containing milt was labelled and kept in between gel ice packs. Then it was transferred to cold handling unit (Gilgal Instruments, Bangalore) which was maintained at 6-10 °C.

The collected milt was analysed under NIKON E360 microscope under phase contrast at 200X magnification for its initial spermatological parameters such as sperm density, motility duration and percentage of live and dead cells. Suitable motility scores were also awarded based on the observation. Sperm density was estimated using hemocytometer (NAUBAEUR, Germany) [20]. Motility duration was evaluated under microscope by placing 1 µl of diluted milt sample and 1 µl of tap water on a grease free glass slide. Duration of motility was estimated as the time elapsing from water addition to the decrease of motility to about half of the original value [21] and the duration was determined in seconds using stopwatch. Motility score was assigned to each sample [22] (Table 1).Percentage of live and dead cells was estimated using Eosin-Nigrosin stain [23-25].

Table 1: Motility score [22]

Criteria	Motility score
All spermatozoa progressively motile with various flagella movements.	5
Most spermatozoa progressively motile with various flagella movements. All other spermatozoa exhibiting strong vibrations *in loco*.	4
Most spermatozoa progressively motile, all others vibrating *in loco*.	3
Most spermatozoa vibrating *in loco*. All others exhibit progressive motion.	2
Most spermatozoa vibrating *in loco*. A few progressively motile.	1
Most spermatozoa immotile, a few slightly vibrating and very few with progressive motion.	0.75
Most spermatozoa immotile with very few slightly vibrating and an occasional one in progressive motion.	0.5
Most spermatozoa immotile with an occasional one slightly vibrating.	0.25
All spermatozoa immotile.	0

The total volume of milt collected was 2 ml and it was divided into two parts. The milt was diluted with 0.85% physiological saline solution and 15% DMSO (85:15) at a ratio of 1:100. One part of diluted milt was kept in the refrigerator at 4 ^{0}C and observations on their spermatological parameters were done at 24 h interval upto 120 h. The remaining part of milt was equilibrated for 10 mins [26] and the diluted milt was loaded into 0.5 ml straws (IMV French Straws). The filled straws were sealed with polymer powder (IMV, France) and were dipped in cold water for proper sealing. The sealed straws were rapidly frozen in LN_2 vapour for 5 mins [27] by keeping the straws above LN_2 at 5 cm. Then the frozen straws were stored in SS canisters in a BA11 cryocan (IBP, India) containing LN_2. Observations on their spermatological parameters were made after 5 days of cryopreservation and it was compared with the observations of the short term preserved spermatozoa. Data were subjected to statistical analysis (paired "t" test) to find out the level of significance.

3. Results and Discussion

The density of the spermatozoa was found to be 2.8×10 [9] sperm cells/ml. The initial mean motility duration of the milt was 151.2 ± 9.67 s. There was a steady decrease in the motility duration and motility score which was preserved in refrigerator as it can be seen in Table 2&3 respectively. The motility duration dropped to 80±7.96 s on 5th day.

Table 2: Mean motility duration of *Cyprinus carpio* spermatozoa preserved under different conditions

S. No.	Type of preservation	Mean motility duration (secs)				
		0 h	24 h	48 h	96 h	120 h
1	Short term preservation	151.2 ± 9.67	126 ± 22.74	109.8 ±14.20	101.6 ±10.06	80±7.96
2	Cryopreservation	151.2 ± 9.67	-	-	-	125±4

Similar reduction in motility duration and score were observed in the spermatozoa of *Tor khudree* stored at 4 °C. They also stated that the motile state of spermatozoa can be maintained only for 4-5 days at 4 °C [28]. Therefore the observation in the present study is falling in line with the observations done earlier.

The motility duration of the cryopreserved spermatozoa was found to be 125±4 s on the 5th day of cryopreservation.The decline in the motility duration from the initial value is generally an accepted phenomenon for all the fish

spermatozoa under cryopreservation [29-33]. There was 47% reduction in the motility duration in short term preserved spermatozoa while the cryopreserved spermatozoa suffered only 17% reduction in their motility duration. Comparatively there was less injuries and more spermatozoa are viable at the end of 5th day under cryopreservation than under short term preserved as it is evident from Fig 1. The difference in the motility duration after 120 h of cryopreservation was significantly different from that of short term preservation at 0.5 level when they were statistically analysed with paired "t" test.

Table 3: Mean motility score of *C. carpio* spermatozoa preserved under different conditions

S. No	Type of preservation	Mean motility score				
		0 h	24 h	48 h	96 h	120 h
1	Short term preservation	5	4	1	1	0.25
2	Cryopreservation	5	-	-	-	3

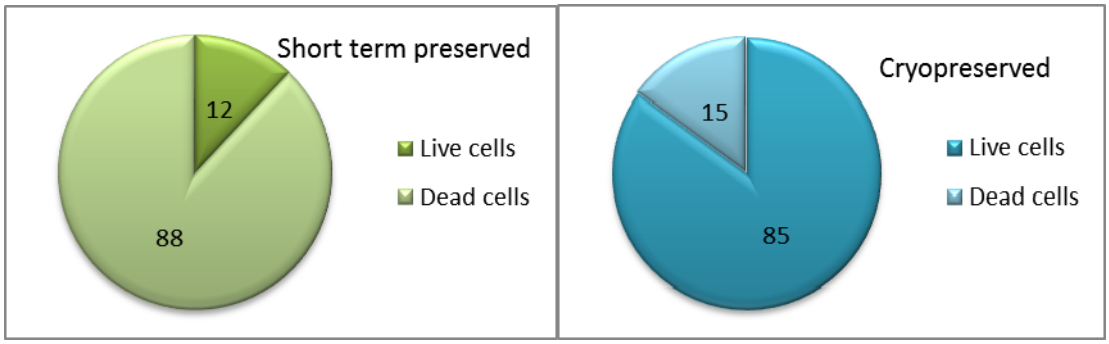

Fig 1: Percentage of live and dead spermatozoa of *C. carpio* during short term preservation and cryopreservation

4. Conclusion

In general, there has been widespread support for the short term preservation i.e. at chilled temperature of 4 °C as it was less cumbersome and economical [5-7, 28]. But the viability of the spermatozoa after a period of 5 days is a limiting factor in the case of short term preserved spermatozoa as it is evident from the present experiment. Although cryopreservation is cumbersome and little expensive, the longevity of the spermatozoa or long term preservation of the spermatozoa is possible only through cryopreservation.

5. Reference

1. FAO. Fishery and Aquaculture Statistics. Food and Agriculture Organization of the United Nations, Rome, 2012.
2. Bromage NR, Roberts RJ. Preservation of gametes. In: Broodstock management and egg and larval quality (eds. Bromage NR and Roberts RJ), Cambridge University Press, Cambridge, 1995, 53-75.
3. Dushkina LA. Viability of herring (*Clupea*) eggs and fertilizing capacity of herring sperm stored under various conditions. J Ichthyol 1975; 15:423-429.
4. Stoss J, Geries L, Holtz W. The role of sample depth in storing chilled rainbow trout (*Salmo gairdneri*) semen under oxygen. Aquaculture 1987; 61:275-279.
5. Saad A, Billard R, Theron, MC, Hollebeq MG. Short term preservation of carp (*Cyprinus carpio*) semen. Aquaculture 1988; 71:133-150.
6. Akash N. Preservation of gametes and fertilization methodology in fringe-lipped carp, *Labeo fimbriatus* (Bloch). MFSc thesis, University of Agricultural Sciences, Bangalore, India, 2001, 54.
7. Agarwal NK, Vandana S, Raghuvanshi SK. Characterization and short-term storage of semen of a coldwater himalayan fish species. Biojournal 2013; 8(1):1-8.
8. Cloud JG, Miller WH, Levanduski MJ. Cryopreservation of sperm as a means to store salmonid germplasm and to transfer genes from wild fish to hatchery populations. Progressive Fish Culturist 1990; 52:51-53.
9. Blaxter PC. Sperm storage and cross fertilization of spring and autumn spawning herring. Nature 1953; 172:1189-1190.
10. Henderson NE, Dewar JE. Short term storage of brook trout milt. Prog Fish Cult 1959; 21:169-171.
11. Kurokura H, Hirano R, Tomita M, Iwahashi M. Cryopreservation of carp sperm. Aquaculture 1984; 37:267-273.
12. Glogowski J, Ciereszko R, Dabrowski K. Cryopreservation of Muskellunge and Yellow Perch semen. North American J Aquaculture 1999; 61:258-262.
13. Akcay E, Bozkurt S. Cryopreservation of mirror carp sperm. Turkion J Veter Ani Sci 2004; 28:837-843.
14. Mohanraj P. Effect of cryopreservation on the irradiated spermatozoa of *Cyprinus carpio* (Linn.). M.F. Sc. Thesis, 2009, 71P.
15. Betsy CJ. Role of supplemented energy sources on spermatological parameters of selected cultivable carps. M.F. Sc. Thesis, 2013, 101P.
16. Bozkurt Y, Ogretmen F, Secer FS. Effect of different extenders and storage periods on motility and fertilization success of Grass Carp (*Ctenopharyngoden idella*) sperm during spawning season. Tarim Bilimleri Dergisi 2009; 15(3):277-284.
17. Billard R, Cosson J, Perchec G, Linhart O. Biology of sperm and artificial reproduction in carp. Aquaculture 1995; 124:95-112.
18. Lahnsteiner F, Patzner RA. Sperm motility of the marine teleosts *Boops boops*, *Diplodus sargus*, *Mullus barbatus* and *Trachurus mediterraneus*. J Fish Biol 1998; 52(4):726-742.

19. Lubzens E, Daube N, Pekarsy I, Magnus Y, Cohen A, Yusefovich F, Feigin P. Carp (*Cyprinus carpio* L) spermatozoa cryobanks-strategies in research and application. Aquaculture 1997; 155:13-30.
20. Hafez ESE. Reproduction in Farm Animals. Edn 5, Lea and Febiger, Philadelphia, USA, 1987, 315-481.
21. Babiak I, Glogowski J, Luezynski MJ, Kucharezyk D, Luezynski M. Cryopreservation of the milt of the northern pike. J Fish Biology 1995; 46(5):819-828.
22. Guest WC, Avault JW, Roussel JD. A spermatology study of channel catfish, *Ictalurus punctatus*. Trans Am Fish Soc 1976; 105:463-468.
23. Blom E. A one-minute live-dead sperm stain by means of eosin-nigrosin. Fertil Steril 1950; 1:176-177.
24. Fribourgh BH. The application of differential staining method to low temperature studies on goldfish spermatozoa. Prog Fish Cult 1966; 28:227-231.
25. Chutia IP, Krishna G, Chaudhary A. Biochemical and biometrical analysis of carp milt. Fish genetics and Biodiversity conservation, Nature conservators, Muzaffarnager, 1998, 205-213.
26. Chao NH, Chan WC, Liu KC, Liao IC. The properties of Tilapia sperm and its cryopreservation. J Fish Biol 1987; 30:107-119.
27. Leung LKP, Jamieson BGM. Live preservation of fish gametes, In: Fish evaluation and systematic evidence from spermatozoa. Jamieson BGM Eds., University Press, Cambridge, 1991, 245.
28. Basavaraja N, Hegde SN. Some characteristics and short-term preservation of spermatozoa of Deccan mahseer, *Tor khudree* (Sykes). Aquaculture Research 2005; 36:422-430.
29. Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: What we ask them to survive. Andrology 1990; 11(3):73-88.
30. Ohta H, Shimma H, Hirose K. Relationship between fertility and motility of cryopreserved spermatozoa of the amago salmon *Oncorhynchus masou* ishikawae. Fisheries Science 1995; 61(5):886-887.
31. Chao NH, Liao IC. Cryopreservation on finfish and shellfish gametes and embryos. Aquaculture 2001; 197:161-189.
32. Bhavani CN, Noble D, Gopalakrishnan A, Sanil NK. Cryopreservation of spermatozoa in the mullet, *Liza parsia:* Effect of cryoprotectants on morphology and motility. J Mar Biol Ass India 2003; 45(1):38-46.
33. Muchlisin ZA. Review: Current Status of Extenders and Cryoprotectants on Fish Spermatozoa Cryopreservation. Biodiversitas 2005; 6(1):66-69.