Indian J. Fish., 62(4) : 140-143, 2015

Note

Effect of egg yolk on enhancing quality of mrigal (*Cirrhinus mrigala*) spermatozoa during cryopreservation

C. JUDITH BETSY AND J. STEPHEN SAMPATH KUMAR

Department of Inland Aquaculture, Fisheries College and Research Institute, Thoothukudi, Tamil Nadu - 628 008, India
e-mail: jstephenkumar@gmail.com

ABSTRACT

The present study was aimed at evaluating the role of egg yolk in maintaining the spermatological parameters of cryopreserved milt of the Indian major carp, *Cirrhinus mrigala*. Egg yolk was supplemented in the cryopreservation medium at three different concentrations *viz*., 5% (T_1), 10% (T_2) and 15% (T_3). During the 42 days of cryopreservation, observations were made once in 7 days on motility parameters based on which the spermatological parameters in terms of motility duration, score, pattern and percentage were determined. The results were statistically analysed using paired 't' test. Samples of milt cryopreserved with egg yolk at 10% (T_2) concentration had significantly higher ($p>0.025$) motility duration (145±4.0 sec) than that of control (83.33±4.5 sec).

Keywords: *Cirrhinus mrigala*, Cryopreservation, Egg Yolk, Energy supplements, Spermatological parameters

World food fish production through aquaculture during the year 2012 was 66.63 million t, of which *Cirrhinus mrigala* contributed 0.4 million t (FAO, 2014). Being an omnivore and tolerant to different water quality conditions, *C. mrigala* is considered as a suitable candidate species for aquaculture in India. It is a monsoon breeder with peak breeding during May to September. Failure or delayed monsoon is known to affect the breeding of this species and a technology that ensures seed production throughout the year is felt necessary. Cryopreservation of gametes is one such technology that can help in tiding over the non-availability of gametes due to monsoon failure/during off-season. Motility of spermatozoa is considered as an essential quality for successful fertilisation. During the course of cryopreservation, cellular reserves of energy get exhausted and eventually spermatozoa cease swimming (Christen *et al.,* 1987; Perchec *et al.,* 1995). It has been reported that fish spermatozoa are capable of using exogenous energy sources only to a limited extent (Mounib, 1967; Harvey and Kelley, 1984), energy supplementation with the sperm fluid, as done for cryopreservation of spermatozoa in other animals holds potential for fish spermatozoa too. Therefore, an experiment was conducted by supplementing egg yolk as an energy source for cryopreservation of mrigal spermatozoa.

Egg yolk is a non-permeating cryoprotectant that can be used in cryopreservation of gametes. It has been reported to be useful when supplemented in extenders and cryoprotectants in many cryopreservation studies. Alvarez and Storey (1993) stated that addition of egg yolk proteins to the freezing medium aids in the recovery of sperm motility. Piironen and Hyvarinen (1983) found that the addition of egg yolk in extender for sperm cryopreservation increased the fertilisation rates in brown trout *Salmo trutta* morpha *lacustris* L. Baynes and Scott (1987) observed higher post-thaw fertility in milt frozen with 10% hen's egg yolk. According to Babiak *et al.* (1995) addition of hen's egg yolk at 10% (v/v) with the Erdahl and Graham's extender increased the hatching success of cryopreserved sperm of pike.

Harvey (1983) compared the effect of egg yolk with methanol and dimethyl sulphoxide (DMSO) in the milt of *Sarotherodon (=Oreochromis) mossambicus* and obtained 10% motility when 5% methanol and 15% egg yolk (v/v) were used, whereas 30% motility was observed when 5% DMSO and 15% egg yolk (v/v) were used. Alderson and MacNeil (1984) reported higher percentage of thawed sperm when diluents contained 10% (v/v) fresh hen's egg yolk. Keeping the above findings in view, the present study was undertaken.

Adult *C. mrigala* males (average body weight 1400 ± 300 g) were selected as milt donors. Milt was collected from 5 males in a sterile, pre-labeled 1.5 ml cryovial as prescribed by Lubzens *et al.* (1997). Milt oozing out during the first 3 to 4 stripping alone was collected. The vials were kept in an icebox cooled with gel ice at 6 to10^{o}C and then transferred to a cold handling chamber (Gilgal Instruments, Bangalore) which was maintained at 5^{o}C, for further processing. The spermatological parameters of the milt collected were recorded prior to processing. The milt was diluted 1:100

with 0.85% physiological saline and DMSO (85:15). The total volume of milt collected was 1.5 ml. This volume was divided into 4 portions and egg yolk was added (v/v) at 3 different concentrations *i.e*., 5% (T_1), 10% (T_2) and 15% (T_3) and a control (C) was also maintained. The diluted milt was equilibrated for 10 min at 5˚C in cold handling chamber (Chao *et al*., 1987; Sarder *et al*., 2009). The equilibrated milt was loaded into 0.25 ml IMV French straws and sealed with a polymer powder (IMV, France). Rapid freezing of straws was done for 5 min (Leung and Jamieson, 1991) by freezing straws in LN_2 vapour by placing the loaded straws at 5 cm above the LN_2 level. They were then immersed in LN_2 in the cryocans. Ten straws from each treatment were taken at each sampling and thawed at 30°C for 30 sec in serological water bath. The thawed milt was observed for the spermatological parameters.

Motility scores were assigned as per the method of Guest *et al.* (1976). Sperm density was estimated using haemocytometer (Neubauer, Germany) as described by Hafez (1987). Motility duration was estimated as described by Babiak *et al.* (1995) and the duration was determined in seconds using stopwatch. Motility pattern was classified into forward movement (+++), circular movement (++) and vibratory movement (+) as per the descriptions of Nomura (1964). Percentage of live and dead cells was estimated using Eosin-Nigrosin stain (Fribourgh, 1966; Chutia *et al.,* 1998). The data were statistically analysed by Paired 't' test.

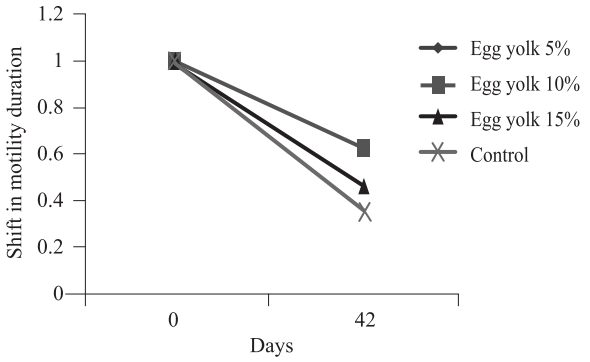

Fig. 1. Rate of change in motility duration of *C. mrigala* spermatozoa diluted and cryopreserved with egg yolk at different concentrations

The mean initial motility duration of milt of *C. mrigala* treated with egg yolk at different concentrations was 230.66±7.5 sec. Spermatozoa cryopreserved with egg yolk at 10% (T_2) showed the highest motility duration of 145±4.0 sec on the 42nd day, whilst egg yolk at 15% (T_3) had motility duration of 106.33±2.0 sec. The motility duration of spermatozoa in control was 83.33±4.5 sec on 42nd day. The motility duration obtained with egg yolk at 10% concentration (T_2) was significantly higher ($p>0.1$) when compared with that of control. Motilty duration was significantly higher ($p>0.025$) when egg yolk at 10% (T_2) concentration was compared with egg yolk at 5% concentration (T_1) (Table 1). It is evident from Fig. 1. that the rate of change of motility duration in T2 and T3 was slower than that of C and T_1 indicating their ability to retain the motility as the cryopreservation duration prolongs.

Initially the spermatozoa in all treatments including the control had a motility score of 5. On day 42, it came down to 1 in control and 2 in samples containing egg yolk (Table 2). The motility pattern of all the treatments including control exhibited forward movement till day 21. From day 28, change in the pattern was observed in control as well as in samples containing egg yolk at 10 (T_2) and 15% (T_3) concentrations. But the motility pattern of spermatozoa cryopreserved with egg yolk at 5% concentration (T_1) was uniform. Similarly, on day 42 of observation, the percentage of live cells was 78 and 73% in the samples cryopreserved with egg yolk at 5% (T_1) and 10% (T_2) respectively.

The change in the motility score was in accordance with the results obtained by Doi (1982). The highest live cells of 78% was noticed with 10% egg yolk (T_2). This was in accordance with Kruger *et al*. (1984) who found that the mean percentage of live sperm was in the range of 80-97% in *C. carpio* and Mohanraj (2009) who reported the mean percentage of live sperm in *C. carpio* to be 88%. Percentage of live cells came down to 63% in the control, making fertilisation a difficult process.

Table 1. Mean values of motility duration (sec) observed at regular intervals in *C. mrigala* spermatozoa cryopreserved with egg yolk at different concentrations

Treatment	Days of cryopreservation							Mean values
	0	7	14	21	28	35	42	
Egg yolk 5% (T_1)	230.66± 7.5	124.33± 3.5	131.33± 7.0	133.66± 3.0	145.66± 3.5	144±4.0	144±3.0	150.52
Egg yolk 10% (T_2)	230.66± 7.5	129±2.0	133.66±4.0	138±3.0	147.66± 3.0	144±3.6	145±4.0	152.56*
Egg yolk 15% (T_3)	230.66± 7.5	87±4.0	88.66±2.5	92.66±4.0	102.33± 4.1	105.33± 2.5	106.33± 2.0	116.13
Control (C)	230.66± 7.5	167±6.5	103.66± 6.0	102.66±6.0	91.33± 1.5	90.33± 1.5	83.33± 4.5	124.13

*(P> 0.025)

Table 2. Motility pattern observed at regular intervals in *C. mrigala* spermatozoa cryopreserved with egg yolk at different concentrations

Treatment	Days of cryopreservation						
	Initial	7	14	21	28	35	42
C	+++	+++	+++	+++	++	++	+
T_1	+++	+++	+++	+++	+++	+++	+++
T_2	+++	+++	+++	+++	+++	+++	++
T_3	+++	+++	+++	+++	++	++	+

However, this could be overcome by supplementation of egg yolk. Supplementation of egg yolk at 10% during cryopreservation of *C. mrigala* spermatozoa helped to increase the percentage of live cells as well as their motility duration so that the cryopreserved spermatozoa is more suitable for artificial fertilisation.

In the present experiment, better spermatological parameters were noticed when egg yolk was supplemented with the milt during cryopreservation. Among the 3 different concentrations tried for egg yolk supplementation, use of egg yolk at 10% concentration gave the best result. The real mechanism of egg yolk in enhancing motility of the spermatozoa of *C. mrigala* needs further investigation.

Acknowledgements

The University authorities of Tamil Nadu Fisheries University are thanked for the support rendered in conducting the experiment as part of M. F. Sc. dissertation work.

References

Alderson, R. and MacNeil, A. J. 1984. Preliminary investigations on cryopreservation of milt of Atlantic salmon (*Salmo salar*) and its application to commercial farming. *Aquaculture,* 43: 351-354.

Alvarez, J. G. and Storey, B. T. 1993. Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in cryopreserved human sperm: glycerol and other polyols as sole cryoprotectant. *J. And.,* 14: 199-209.

Babiak, I., Glogowski, J., Luezynski, M. J., Kucharezyk, D. and Luezynski, M. 1995. Cryopreservation of the milt of the northern pike. *J. Fish Biol.,* 46: 819-828.

Baynes, S. M. and Scott, A. P. 1987. Cryopreservation of rainbow trout spermatozoa: the influence of sperm quality, egg quality and extender composition on post-thaw fertility. *Aquaculture,* 66: 319-354.

Chao, N. H., Chan, W. C., Liu, K. C. and Liao, I. C. 1987. The properties of tilapia sperm and its cryopreservation. *J. Fish Biol.,* 30: 107-119.

Christen, F., Gatti, J. L. and Billard, R. 1987. Trout sperm motility: the transient movement of trout sperm is related to changes in the concentration of ATP following the activation of flagellar movement. *Eur. J. Bioch.,* 166: 667-671.

Chutia, I. P., Krishna, G. and Chaudhary, A. 1998. Biochemical and biometrical analysis of carp milt. In: Ponniah, A. G., Das, P. and Verma, S. R. (Eds.), *Fish genetics and biodiversity conservation.* Nature conservators, Muzaffarnager, p. 205-213.

Doi, M., Hoshino, T., Taki, Y. and Ogasawara, Y. 1982. Activity of the sperm of the bluefin tuna *Thunnus thynnus* under fresh and preserved conditions. *Bull. Japan Soc. Sci. Fish.,* 48: 495-498.

FAO 2014. *Fishery and aquaculture statistics*. Food and Agriculture Organization of the United Nations, Rome, 46 pp.

Fribourgh, B. H. 1966. The application of differential staining method to low temperature studies on goldfish spermatozoa. *Prog. Fish Cult.,* 28: 227-231.

Guest, W. C., Avault, Jr. J. W. and Roussed, J. D. 1976. Preservation of channel catfish sperm. *Trans. Am. Fish. Soc.,* 105: 469-474.

Hafez, E. S. E. 1987. *Reproduction in farm animals,* 5th edn. Lea and Febiger, Philadelphia, USA, p. 315-481.

Harvey, B. 1983. Cryoproservation of *Sarotherodon mossambicus* spermatozoa. *Aquaculture,* 32: 313-320.

Harvey, B. and Kelley, R. N. 1984. Chilled storage of *Sarotherodon mossambicus* milt. *Aquaculture,* 36: 85-95.

Kruger, J. C. D. W., Smit, G. L., Vuren, J. H. J. V. and Ferreiera, J. T. 1984. Some chemical and physical properties of the semen of *Cyprinus carpio* L. and *Oreochromis mossambicus* (Peters). *J. Fish Biol.,* 24: 263-272.

Leung, L. K. P. and Jamieson, B. G. M. 1991. Live preservation of fish gametes. In: Jamieson, B. G. M. (Ed.), *Fish evaluation and systematic evidence from spermatozoa.* University Press, Cambridge, 245 pp.

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

Mohanraj, P. 2009. Effect of cryopreservation on the irradiated spermatozoa of *Cyprinus carpio* (Linn.). M.F.Sc. Thesis,

Tamil Nadu Veterinary and Animal Sciences University, Chennai, 71 pp.

Mounib, M. S. 1967. Metabolism of pyruvate, acetate and glycoxylate by fish sperm. *Comp. Biochem. Physiol.,* 20: 987-992.

Nomura, M. 1964. Studies on reproduction of rainbow trout *Salmo gairdneri* with special reference to egg takens VI - the activities of spermatozoa in different diluents and preservation of semen. *Bull. Japan Soc. Sci. Fish.,* 30: 723-733.

Perchec, G., Jeulin, C., Cosson, J., Andre, F. and Billard, R. 1995. Relationship between sperm ATP content and motility of carp spermatozoa. *J. Cell Sci.,* 108: 747-753.

Piironen, J. and Hyvarinen, H. 1983. Cryopreservation of spermatozoa of the white fish, *Coregonus muskun* Pallas. *J. Fish Biol.,* 22: 159-163.

Sarder, M. R. I., Rafiquzzaman, S. M., Sultana, R. and Faridul Islam, M. 2009. Cryopreservation of spermatozoa of mrigal, *Cirrhinus cirrhosus* with a view to minimise inbreeding and hybridisation. *J. Bangl. Agri. Univ.,* 7: 211-215.

Date of Receipt : 11.03.2015
Date of Acceptance : 20.08.2015