



Role of glucose in enhancing life and potency of *Cirrhinus mrigala* spermatozoa during cryopreservation

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Abstract: Cryopreservation of fish gametes is an emerging technology and breeding with cryopreserved gametes is advancement in fish seed production. Success of cryopreservation is evaluated by the post - thaw motility of the spermatozoa, an for which energy is required. Cryopreservation is known to cause changes in the seminal plasma that would alter the energy supply for the motility of the spermatozoa. Therefore, energy supplementation is found to be useful during cryopreservation. *Cirrhinus mrigala* spermatozoa were cryopreserved along with glucose as a co-cryoprotectant after 1:100 dilutions with 0.85% physiological saline as extender and Dimethyl Sulfoxide (DMSO) as cryoprotectant (85:15). The diluents contained glucose at four different concentrations, viz., T₁ (0.25%), T₂ (0.5%), T₃ (0.75%) and T₄ (1%). The diluted milt was equilibrated for 10 min at 5° C and loaded into 0.25 ml straws. The loaded straws were then frozen with LN₂ vapour for 5 min and immersed in liquid nitrogen. Observations were made once in 7 days for 42 days on motility parameters based on which the duration, score, pattern and percentage were determined. The spermatozoa cryopreserved with glucose at 0.5% concentration showed the highest motility duration of 204±3.6 s whereas Control group showed motility duration of only 83.33± 4.5 s on 42nd day. The difference in motility duration was statistically significant (P>0.025). The present study revealed the benefits of adding glucose at 0.5% during cryopreservation as it could help in maintaining the motility duration and survival of spermatozoa.

Keywords: *Cirrhinus mrigala*, Cryopreservation, Energy supplements, Glucose, Spermatological parameters

INTRODUCTION

World aquaculture production during the year 2012 was 90.43 million tonnes and the production of food fish has increased from 59.9 million tonnes in 2010 to 66.63 million tonnes in 2012 (FAO, 2014). It is worth mentioning that 9.41 kg of food fish per person in the world for consumption has been supplied by aquaculture (FAO, 2014). This shows the potential of aquaculture as a possible enterprise to provide food security for the future generations.

Carp are preferred worldwide for their potential culture qualities and marketability. India is known as a “Carp Country” where among other fishes; Indian major carps contribute significantly for the fish production in the country. *C. mrigala* has been one of the principle cultured species in India. Being an omnivore and tolerant to different water quality conditions, it has been considered as a good candidate species for aquaculture in India. It is a monsoon breeder having peak breeding during October to December due to copious river flow and nutrient loading due to surface run off.

Breeding of *C. mrigala* is severely influenced by the monsoon failure and hence there is a need for a technology which must ensure seed production throughout the year. Cryopreservation of gametes can help in solving

this problem.

Reports on the success of cryopreservation of fish spermatozoa have been made on different fishes (Blaxter, 1953; Henderson and Dewar, 1959; Withler and Morley, 1968; Stein, 1975; Kurokura *et al.*, 1984; Glogowski *et al.*, 1999; Akcay and Bozkurt, 2004; Mohanraj, 2009; Betsy, 2013). Motility is considered as the major factor determining the quality of the spermatozoa (Billard *et al.*, 1995; Lahnsteiner and Patzner, 1998). Since motile spermatozoa can effectively aid in the fertilization of the eggs, it is necessary to retain the motility as maximum as possible in order to have the successful fertilization. Energy will be spent for the flagella movement, and therefore spermatozoa cease swimming when the cellular reserves of energy are exhausted (Christen *et al.*, 1987; Perchec *et al.*, 1995). Hence, supplementing energy is considered as a possibility to encourage the motility of spermatozoa even after cryopreservation.

It has been shown that fish spermatozoa are capable of using exogenous energy sources to a limited extent (Mounib, 1967; Harvey and Kelley, 1984). One of the energy sources studied is glucose which is a non-permeating co-cryoprotectant.

Glucose is the chief substance utilized for energy in the fish semen (Hamner and Charles, 1969). Glucose is used as an external cryoprotectant (Purdy, 2006) and as a

non-ionic immobilizer in freshwater fish (Horváth *et al.*, 2003; Cruz-Casallas *et al.*, 2004; Viveiros and Godinho, 2009).

Stoss and Refstei (1983) found a very simple extender consisting of 0.3M glucose and 10% DMSO to be the most successful of a number of extenders in cryopreservation of the sperm of Atlantic salmon and sea trout which might support the concept of glucose supporting the spermatozoa. Longer motility in the spermatozoa of guppy was noticed when extracellular glucose was given (Gardiner, 1978). Similarly addition of glucose to the extender had enhanced the quality of milt and the storage duration (Ponniah *et al.*, 1999). In *Xiphophorus helleri*, the addition of glucose yielded higher and longer motility duration for fresh and cryopreserved sperm while the concentration was kept lesser than 11.0 mM. The effect was not seen when the concentration of glucose was higher than 11.0 mM (Dong *et al.*, 2006).

Similar application of glucose in the cryopreservation of fish gametes is seen with other fishes too. Bozkurt *et al.* (2005) reported use of glucose at 300 mM during cryopreservation of rainbow trout spermatozoa. The toxicity of cryoprotectant is reduced by the addition of glucose (Melo and Godinho, 2006). Grace (2013) stated that when monosaccharide glucose was added to the cryoprotectant, the post-thaw survival rate of *Arius arius* spermatozoa was increased due to the energy supplying nature of glucose. Therefore, the present study aims at identifying the optimal concentration of glucose in the diluents for the cryopreservation of fish milt and its influence on the spermatological parameters like percentage of motile spermatozoa, motility duration, motility score and motility pattern.

MATERIALS AND METHODS

Adult *Cirrhinus mrigala* males with average body weight of 1400±300g were collected after checking their maturity. Milt was collected from 3 males as prescribed by Lubzens *et al.* (1997). The genital pore area was cleaned with double ply tissue paper to remove moisture and mucus and gentle pressure was applied in the abdomen to collect the oozing milt. The milt was collected in a labeled cryovial (1.5ml cap). Milt oozing out during the first three to four stripping alone was collected. The vials were kept in the icebox cooled with gel ice at 6 to 10°C and then transferred to cold handling chamber (Gilgal Instruments, Bangalore) which was maintained at 5°C for further processing. The spermatological parameters of the milt collected were recorded prior to processing.

The collected milt was pooled together and the milt was diluted to 100 folds with 0.85% physiological saline solution and DMSO (85:15). The total volume of milt collected was 1.5 ml which was divided into 5 portions and glucose were added (w/v) at 4 different concentrations viz, T₁ (0.25%), T₂ (0.5%), T₃ (0.75%) and T₄ (1%) and a Control (C) was 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 0.25ml plastic straws and sealed with a polymer powder (IMV, France). Rapid freezing of straws was done for 5 minutes (Leung and Jamieson, 1991) by freezing straws in LN₂ vapour. The distance between the straws and LN₂ was maintained at 5cm. They were then transferred to BA11 cryocans containing LN₂. Observations were made once in 7 days for 42 days. Ten straws from each treatment were taken at each sampling and thawed at 30°C for 30s in serological water bath. The thawed milt was observed for the spermatological parameters like motility duration, motility pattern, motility score and percentage of live and dead cells.

The collected milt was observed using NIKON E360 microscope under phase contrast at X 200 magnification. The initial spermatological parameters such as sperm density, motility duration and percentage of live and dead cells were observed and recorded. Suitable motility scores were assigned based on the observation following Guest *et al.* (1976). Motility duration was evaluated under microscope by placing 1µl of diluted milt sample and 1µl of tap water on a grease free glass slide and estimated as described by Babiak *et al.* (1995) and the duration were 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 as described by Fribourgh (1966) and Chutia *et al.* (1998). The data were statistically analyzed by paired t test.

RESULTS

The mean initial motility duration of the spermatozoa of *C. mrigala* was 230.66±7.5s. There was a decrease in the motility duration when observed on 7th day after which the duration increased slowly. The increase in motility duration remained constant on 42nd day. Spermatozoa cryopreserved with glucose at 0.5% had the highest motility duration of 204±3.6s, whereas, that of glucose at 0.75% had the lowest motility duration of 121±2.0s on 42nd day. Spermatozoa in the Control had motility duration of 83.33±4.5s on 42nd day. All the samples cryopreserved with glucose at different concentrations had relatively higher percentage of motility duration than Control (Table 1). The values observed with the spermatozoa cryopreserved with glucose at 0.5% concentration was statistically significant (P>0.025) when compared with Control.

When the motility score is assigned for the observation, it is understood that spermatozoa of all treatments including the Control had a sharp reduction in the motility score after 14th day. It came down to 1 on 42nd day for the Control but the samples contained glucose at different concentrations remained at 2 on 42nd day vouching the fact that the glucose could support the motility better

Table 1. Mean values of motility duration (s) observed at regular intervals with *C. mrigala* spermatozoa diluted and cryopreserved with glucose at different concentrations.

Treatment	Days of cryopreservation							Mean values
	0	7	14	21	28	35	42	
Glucose 0.25% (TG ₁)	230.66±7.5	174±2.6	102±5.5	108.33±3.1	122.33±3.0	128.66±6.0	122.33±3.0	141.18
Glucose 0.5% (TG ₂)	230.66±7.5	159.66±2.5	180.33±7.1	182.33±10.1	183±3.4	183.33±9.2	204±3.6	189.04*
Glucose 0.75% (TG ₃)	230.66±7.5	159±4.0	109±4.5	111.33±2.5	117±2.0	117.33±2.1	121±2.0	137.90
Glucose 1% (TG ₄)	230.66±7.5	151.33±3.0	123.33±2.5	135.66±2.5	159.66±3.0	175.33±3.5	165.66±3.1	163.09
Control	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

Level of significance *(P> 0.025)

Table 2. Mean values of motility score observed at regular intervals in *C. mrigala* spermatozoa cryopreserved with glucose at different concentrations.

Treatment	Days of cryopreservation						
	Initial	7	14	21	28	35	42
Control	5	4	4	3	2	2	1
Glucose 0.25%	5	4	3	3	3	3	2
Glucose 0.5%	5	4	4	4	3	3	2
Glucose 0.75%	5	4	4	4	3	3	2
Glucose 1%	5	4	4	4	3	3	2

(Table 2 and Fig. 1).

The observations on the motility pattern also indicated that the spermatozoa cryopreserved with glucose at 0.25% and 0.5% had been uniform with forward movement (Table 3). The percentage of live cells was 71% in the samples cryopreserved with glucose at 0.5% (Fig. 2) whereas it was lower for control (63%). Samples containing glucose at 0.75% and 1% concentrations had 64% and 63% of live cells respectively on 42nd day of observation.

DISCUSSION

C. mrigala spermatozoa cryopreserved with glucose at 0.5% concentration exhibited a motility duration which is 52% higher than that of Control and the value was

statistically significant (P>0.025). All the samples cryopreserved with glucose at different concentrations had relatively higher percentage of post-thaw motility duration than that of Control (Table 1). As it is seen in fig.1, the rate of change in the motility duration for various doses of glucose in the cryopreserved *C. mrigala* spermatozoa did not show decline. There was distinct change in motility pattern.

The present results fall in line with the reports by other authors. Carolsfeld *et al.* (2003) stated that the post-thaw motility rate was 80-100% when piracanjuba (*Brycon orbignyanus*) sperm was cryopreserved with glucose at 5%. Nascimento *et al.* (2010) reported the greatest post-thaw sperm motility of 81% with fastest sperm velocities when sperm samples of Amazonian freshwater fish

Table 3. Motility pattern observed at regular intervals in *C. mrigala* spermatozoa cryopreserved with glucose at different concentrations.

Treatment	Days of cryopreservation						
	Initial	7	14	21	28	35	42
Control	+++	+++	+++	+++	++	++	+
Glucose 0.25%	+++	+++	+++	+++	+++	+++	+++
Glucose 0.5%	+++	+++	+++	+++	+++	+++	+++
Glucose 0.75%	+++	+++	+++	+++	+++	++	+
Glucose 1%	+++	+++	++	++	++	+	+

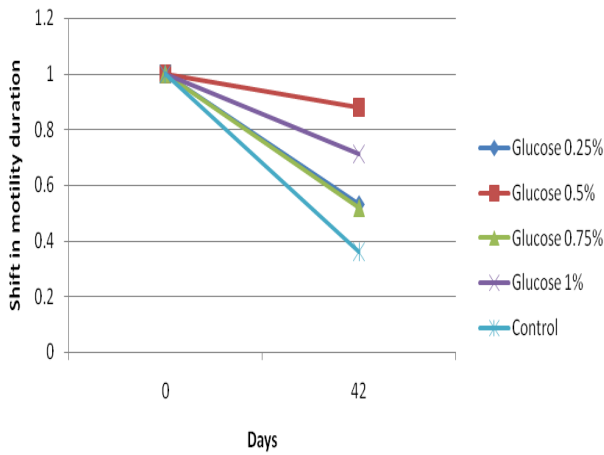


Fig. 1. Rate of change in motility duration of *C. mrigala* spermatozoa diluted and cryopreserved with glucose at different concentrations.

pirapitinga (*Piaractus brachypomus*) were cryopreserved in glucose-methylglycol. Similar result was observed by Viveiros *et al.* (2010) who cryopreserved the spermatozoa of Streaked prochilod (*Prochilodus lineatus*) in glucose and found 75% post-thaw motility. Martinez *et al.* (2012) also observed 71% post-thaw motility when glucose was used at 5% concentration during cryopreservation of bocachico (*Prochilodus magdalenae*).

The observations exhibited in the fig. 1 explain the swing in the motility pattern and duration that has been considered inherent and unavoidable in the cryopreservation of the gametes (Hammerstedt *et al.*, 1990; Ohta *et al.*, 1995; Chao and Liao, 2001). The increased concentration of glucose might have shifted the osmolality of the diluent thereby the motility duration was affected as commented by Hammerstedt *et al.* (1990) and Takai and Morisawa (1995).

The motility score of the cryopreserved spermatozoa showed similar reduction (Table 2). All the treatments including Control exhibited a sharp reduction in the

motility score only after 14th day. While it came down to 1 on 42nd day for the Control, the samples contained glucose at different concentrations remained at 2 on 42nd day. The change in the motility score was in accordance with the results obtained by Doi (1982). The observations on the motility pattern also indicated that the spermatozoa cryopreserved with glucose at 0.25% and 0.5% had been uniform without any alteration excepting on the number of cells.

These observations on motility score and motility pattern lead to the point that when energy supplements are provided, the efficiency of the spermatozoa could be protected in spite of a reduction in the number of motile cells. The motility pattern of the live spermatozoa after cryopreservation had a motility pattern that was useful for the fertilization of the eggs.

Le Roux and Steyn (1968) opined that high percentage of live sperm cells is a prerequisite to fertilize thousands of eggs deposited on a substrate during spawning activities in fishes. In the present study, the percentage of live cells was 71% when the spermatozoa were cryopreserved with glucose at 0.25% and 0.5% concentrations. The result was similar to 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%. It is evident that glucose at concentrations of 0.5% alone can be used in the extender for enhancing the motility duration of the spermatozoa of *C. mrigala*.

Conclusion

In the present study, the motility duration of spermatozoa cryopreserved with glucose at 0.5% concentration showed the highest motility duration of 204±3.6 s. Higher percentage of live cells (71%) and forward movement of spermatozoa were observed when 0.5% glucose was supplemented during cryopreservation. Similar results on motility pattern and motility score were noticed when glucose was used at 0.25% concentration

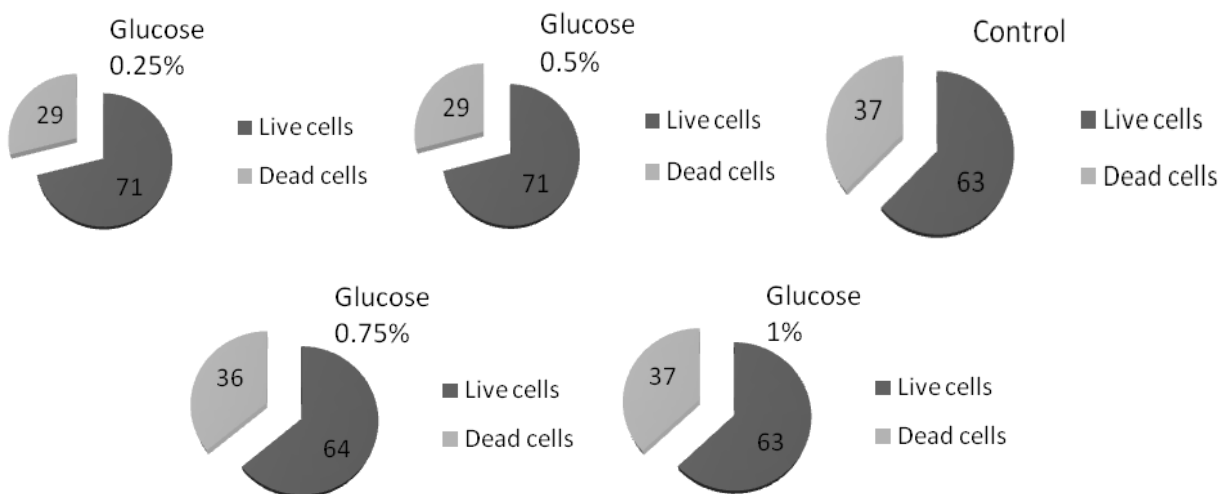


Fig. 2. Percentage of live and dead cells of *C. mrigala* milt cryopreserved with glucose at different concentrations.

also. Hence it can be concluded that energy can be spared from external energy supplements like glucose (at 0.5% w/v) for the spermatozoa activities.

This study would be beneficial for enhancing or protecting the efficiency of the spermatozoa subjected to the cryopreservation.

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