



Development of Milt Cryopreservation Protocol for Economically Important Minor Carp *Cirrhinus reba* (Hamilton, 1822)

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ABSTRACT

Background: Reba carp is one of the promising candidates for species diversification in fresh-water aquaculture. It is available in all the major rivers of India. Cryopreserved milt will be useful in genetic exchange between remotely located farms. For using cryomilt as a tool for genetic exchange, species specific milt cryopreservation protocol is required.

Methods: Milt was cryopreserved using three extenders (7, 7B and 9C) and two cryoprotectants (DMSO and methanol) and evaluated with fresh milt as control. Milt was cryopreserved in 1:6 dilution in French medium straws of 0.5 ml. The feasibility of selected extender and cryoprotectant combinations was assessed by the ability of cryopreserved sperm to fertilize the *C. reba* eggs and hatching percentage. Fertilized eggs were incubated in 0.5 L bowl and a flow-through incubation system. Analysis was done for both bowl and flow-through incubation separately and combined as well.

Result: Better hatching percentage was obtained in flow-through incubation system for all the treatments. In combined analysis extender 7 with 10% DMSO as cryoprotectant gave significantly higher hatching rate (44.5%) and (75%) in comparison to control. Better performance was noticed in flow-through incubation system (50.09%) as compared to bowl (39.1%) in the same treatment. Thus, it can be concluded that extender 7 with 10% DMSO (V/V) is suitable to retain sperm quality in reba carp having optimal sperm motility, high fertility and hatching close to the values obtained with fresh sperm in flow-through incubation system.

Key words: Cryopreservation, Extender, Incubation, Minor carp.

INTRODUCTION

The carps constitute 95% of the total freshwater fish production in India (FAO, 2014) with the major carps being the choice of preference for decades. But recently the freshwater aquaculture system has started to diversify by including the medium and minor carps as well. The natural waters of the country inhabit several minor carp species that show high compatibility with the major carps and have the potential to enhance the biomass yield (Sahu *et al.*, 2007). Studies have revealed that the incorporation of these minor carps in the conventional major carp culture has proved to be an effective strategy to enhance production with high economic return as they fetch 20-30% higher market price as compared to the IMCs (Das and Mishra, 2016). Among these minor carps, reba, has gained popularity among the fish farmers and proved to be a potential candidate species for culture and captive breeding (Ponniah and Sarkar, 2000; Ayyappan and Jena, 2001). The fish is popular for its nutritive value, as a rich source of calcium, protein and low fatty acid (Afroz and Begum, 2014) and relished for its flavour and due to low quantity of spines (Chondar, 1999). Researchers have revealed that the content of protein, carbohydrate and fat provided by *C. reba* is relatively higher than that of the IMCs (Khawaja, 1966; Sharma and Simlot, 1971). Induced breeding of reba was done by Chattopadhyay *et al.* (2013) using ovaprim @ 0.3 ml and 0.5 ml per kg body weight in males and females respectively. Thus, the introduction of this carp in the culture ponds along with major carps may turn out to be a successful step towards commercialization.

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A technique that can be adopted to improve the gene pool of this highly-priced fish is cryopreservation which is the simplest method to preserve the genetic material at ultra-low temperature (-196°C) for a prolonged period. Cryopreservation of germplasm holds immense prospects in the aquaculture industry (Maria and Carneiro, 2012) as the hatcheries can supply seed round the year as per demand. To ensure this, milt cryobanking is considered to be one of the most established and commercialized tools. For success of cryobanking, species specific protocol is required (Diwan *et al.*, 2010) and till date protocol for 200 fish species is reported (Diwan *et al.*, 2020). In this

background we evaluated three existing extenders, two cryoprotectants and two incubation methods for developing species specific fish milt cryopreservation protocol for aquaculture important reba carp.

MATERIALS AND METHODS

Experimental animals

This study was approved by the Animal Ethics Committee of National Bureau of Fish Genetic Resources and was conducted in experimental farm of ICAR-NBFGR, Lucknow during July-Aug, 2020. Sexually mature brooders (100-350 g) were selected for hormonal induction. Hormonal induction was performed via intraperitoneal injections (male: 0.2 ml/kg body weight) of commercial gonadotropin GnRH (Ovarim) to increase milt volume and decrease viscosity, as a routine method for Cyprinids. During administration of hormonal dose, fishes were anaesthetized using 2-Phenoxyethanol (0.4 ml/L) as per Bernáth *et al.* (2016). For hormonal induction of females, intraperitoneal injections were administered at 0.5 ml/Kg of body weight.

Milt collection and quality assessment

Live, running ripe milers (15 nos.) of reba were used for milt collection. After 4 hrs of hormonal induction, milt was collected in dry plastic boxes by applying abdominal pressure in anaesthetized condition. Sperm motility was assessed under a phase contrast microscope at 40 X.

Diluent preparation

Working extender 7 (NaCl 128 mM, KCl 0.0027 mM, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.0014 mM and NaHCO_3 0.0024) 7B (extender 7 with 2% egg yolk) and 9C (NaCl 111 mM, KCl 0.019 mM, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.002 mM, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.008 mM and Glucose 0.003 mM) were prepared and stored in chilled condition. Dimethyl sulphoxide and methanol were used as cryoprotectants. After quality assessment, non-activated sperm cells were pooled and diluted with diluent (extender with 10% cryoprotectant v/v) in dilution rate of 1:6. Cryoprotectants were added to the extender just prior to mixing with milt. French straws (Minitubes, GmbH, Germany) of 0.5 ml capacity were filled with extended milt using manual filling system and equilibrated over ice for 10 min.

Freezing

After equilibration, they were exposed to liquid nitrogen (LN_2) vapour on a freezing stand at 3.0 cm above surface level of LN_2 in styrofoam box. After 10 minutes of holding in liquid nitrogen vapour, the straws were plunged into liquid nitrogen (-196°C) and stored in canisters in cryocan.

Fertility trial

Eggs from running ripe females (4 nos.) were used for fertility trial purpose and it was collected approximately 6 hrs after hormonal induction. Eggs were stripped by applying abdominal pressure and collected individually for each female. Based on visual observation of eggs, all good quality

eggs from different females were pooled and stored in covered condition. To thaw frozen milt, the straws were rapidly plunged into warm water (37°C) with vigorous shaking and time elapsed was recorded. Plastic tubs (10 L) were used for fertilization of measured volume of eggs (1 spoonful approx. 9 ml~16,320±560 eggs) and was calculated as 16,000 for all practical purposes. One spoonful egg containing 16,000 eggs were fertilized with 2 ml (4 numbers of straws of 0.5 ml capacity) cryopreserved milt (preserved for 2 weeks), mixed well with shaking and 5 ml of tap water was added to activate spermatozoa. In control, 0.3 ml fresh milt collected 4 hrs after hormonal induction was added, mixed well and activated using tap water for fertilization of eggs. Fertilized eggs were washed thrice with tap water to remove all milt debris and kept for incubation.

Incubation

For hatching, two types of incubation systems were used viz. bowl and flow through. For hatching estimation, 100-150 eggs were collected randomly and incubated in 0.5 L plastic bowl in replicates. Remaining eggs were incubated in flow-through system in replicates. Hatching estimation was done just prior to hatching. In bowl incubation system, all the eggs were counted. In flow-through incubation system, randomly 100-150 eggs were sampled three times from incubation tray and all the eggs were counted. Hatching took place after 14-16 hrs of fertilization. From the total number of eggs present in bowl/ trough at the start of the trial, numbers of hatchlings were counted to obtain the hatching percentage.

$$\text{Hatching (\%)} = \frac{\text{Hatchlings}}{\text{Total eggs}} \times 100$$

Statistical analysis

Statistical tests were conducted with the IBM SPSS Statistics 26 software. The distribution of the residuals for each model was tested for normality and an arcsine transformation was performed when the data did not follow a normal distribution. The data were subjected to Levene's test of homogeneity of variance. Levene's test was rejected, Welch analysis of variance was applied and difference of means was compared by the Games-Howell test. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Extenders and cryoprotectants play a vital role in cryopreservation and their suitability differs from one species to another (Muchlisin, 2005). The efficacy of the process of cryopreservation is highly increased if the milt suspension is diluted with a suitable diluent (extender and cryoprotectant). Extenders used for milt dilution are generally formulated to be compatible with the physico-chemical composition of the seminal fluid of the candidate species. An ideal extender reversibly inhibits sperm activation and provides suitable pH and isotonic conditions for their survival.

Table 1: Comparison between hatching percentage obtained with different treatments.

Treatment	Bowl Incubation	Flow-through	Combined
	Mean±SD (% relative to control)	Mean±SD (% relative to control)	Mean±SD (% relative to control)
Control	58.70±0.01 ^a	60.44±0.00 ^a	59.60±0.00 ^a
DMSO7	39.10±0.03 ^b (66.6)	50.09±0.07 ^b (82.9)	44.56±0.05 ^b (74.8)
DMSO7B	16.20±0.10 ^c (27.6)	41.08±0.36 ^b (68.0)	27.78±0.28 ^c (46.6)
DMSO9C	10.50±0.01 ^c (17.9)	25.78±0.08 ^b (42.6)	17.50±0.11 ^c (29.4)
Methanol7	12.20±0.18 ^c (20.8)	9.06±0.17 ^c (15.0)	10.56±0.08 ^c (17.7)
Methanol7B	1.40±0.30 ^c (2.4)	4.52±0.94 ^c (7.48)	2.72±0.30 ^c (4.6)
Methanol9C	4.70±0.04 ^c (8.0)	9.19±0.06 ^c (15.2)	6.76±0.04 ^c (11.3)

* The treatment means with significant differences are indicated by different superscripts.

* Hatching percentage relative to control is given in bracket.

Cryoprotectant protects the cells against the formation of intracellular ice crystals and prevents excessive dehydration (Cabrita *et al.*, 2005).

The hatching percentage was used as a parameter for the comparative evaluation of different cryoprotectants and extenders. Between the two cryoprotectants used, 10% DMSO with extender 7 (44.6%) gave significantly better results compared to other combinations of cryoprotectant and extender at the post-thaw level which confirmed its suitability for sperm cryopreservation of the selected minor carp species (Table 1). The results are in accordance to previous observations reported for several freshwater species (Viveiros and Godinho, 2009). Dimethyl Sulfoxide at concentrations of 5-20% (v/v) has been used successfully for cryopreservation of several carps (Routray *et al.*, 2006). While working on cryopreservation in silver carp and grass carp, Chen *et al.* (1992) reported efficiency of DMSO at 10-12%. Dimethyl sulfoxide (DMSO) has been found to show promising results in terms of fertility and motility rates and is considered the cryoprotectant of choice for most of the species (Suquet *et al.*, 2000). According to Anchordoguy *et al.* (1987), the success achieved using DMSO is because of its low toxicity, fast rate of penetration and interaction with sperm membrane phospholipids. As compared to other potential cryoprotectants, DMSO has a higher efflux capacity and a lesser temperature-dependent process of cellular inflow making it one of the best and most preferred cryoprotectant (Huang *et al.*, 2018). Methanol on the other hand, has been found to be more toxic with high permeation capacity (Noble, 2003). Lahnsteiner *et al.* (2004) and Glogowski *et al.* (2002) used methanol as a cryoprotectant for cryopreservation of *Acipenser ruthenus* and *Acipenser baeri* sperm respectively and observed lower motility and fertilization rate than DMSO. While working on cryopreservation of bata carp (*Labeo bata*), Noor *et al.* (2018) reported low post-thaw motility ($\leq 40\%$) with methanol as cryoprotectant in comparison to DMSO (70%). Our results are in accordance with this study. During fish milt cryopreservation protocol development of olive barb (*Puntius sarana*), Nahiduzzaman *et al.* (2011) reported 61% post-

thaw motility with 10% DMSO as cryoprotectant. In the same study, 37% hatching was observed using cryomilt and 62 % hatching with fresh milt was observed. These results agree with our results. On the other hand, methanol was proved to be more efficient cryoprotectant than DMSO in common carp with 63 % post-thaw motility (Horvath *et al.*, 2003).

The comparison of results revealed the optimum extender required for successful milt cryopreservation in reba carp. Among Extenders 7, 7B and 9C, extender 7 resulted in significantly higher hatching *i.e.*, 44.56% with DMSO as cryoprotectant (Table 1). Presence of egg-yolk in extender 7B did not exhibit any improved performance in the hatching rate.

Between the bowl and flow-through incubation systems, the hatching percentage was observed to be higher in the flow-through system for all combinations of extender and cryoprotectant (Table 1). Fertilized eggs of this species easily get settled at bottom and results in poor hatching, so flow-through system is recommended for proper incubation of eggs. This may be attributed to continuous circulation of water providing sufficient oxygen to the eggs along with proper removal of dead and decayed eggs.

CONCLUSION

The results from the present paper demonstrated successful sperm cryopreservation of *Cirrhinus reba* and concluded that extender 7 with DMSO (10% v/v) as cryoprotectant proves to be the best combination for freezing *C. reba* sperm with a hatching success of 44.6%. When results are expressed in comparison to control, it is nearly 75%. For commercialization of sperm cryopreservation protocol of this species, bigger cryovials should be used and bulk fertilization using cryomilt need to be tested. Cryomilt can be used for production of genetically diversified seed for commercial aquaculture of this species and it will help in species diversification.

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