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Sperm cryopreservation of the Indian major carp, *Labeo calbasu*: Effects of cryoprotectants, cooling rates and thawing rates on egg fertilization

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ABSTRACT

A sperm cryopreservation protocol for the Indian major carp, *Labeo calbasu*, was developed for long-term preservation and artificial fertilization. Milt collected from mature male fish were placed in Alsever's solution (296 mOsmol kg^{-1}) to immobilize the sperm. Cryoprotectant toxicity was evaluated by motility assessment with dimethyl sulfoxide (DMSO) and methanol at 5, 10 and 15% concentrations. DMSO was more toxic at higher concentrations than methanol, and consequently 15% DMSO was excluded from further study. A one-step cooling protocol (from 5 to 80 °C) with two cooling rates (5 and 10 °C/min) was carried out in a computer-controlled freezer (FREEZE CONTROL® CL-3300; Australia). Based on post-thaw motility, the 10 °C/min cooling rate with either 10% DMSO or 10% methanol yielded significantly higher ($P = 0.011$) post-thaw motility than the other rate and cryoprotectant concentrations. Sperm thawed at 40 °C for 15 s and fresh sperm were used to fertilize freshly collected *L. calbasu* eggs and significant differences were observed ($P = 0.001$) in percent fertilization between cryopreserved and fresh sperm as well as among different sperm-to-egg ratios ($P = 0.001$). The highest fertilization and hatching rates were observed for thawed sperm at a sperm-to-egg ratio of 4.1×10^5:1. The cryopreservation protocol developed can facilitate hatchery operations and long-term conservation of genetic resources of *L. calbasu*.

1. Introduction

Cryopreservation includes the storage of genetic materials at the temperature of liquid nitrogen (−196 °C) without major change in biological integrity potentially for hundreds or thousands of years. Compared to cattle semen, fish sperm cryopreservation is a developing science with only a rudimentary theoretical framework and often empirical advances made by trial and error. Sperm cryopreservation protocols have been developed for more than 200 finfish and shellfish species around the world (Tiersch et al., 2007). In Bangladesh, research on fish sperm cryopreservation started in 2004 (Hossain et al., 2011), and only 15 species have been studied to date. With respect to the degraded biodiversity of Bangladesh, fish sperm cryopreservation could be employed as an effective strategy to save imperiled species by facilitating the storage of gametes in gene banks (Nahiduzzaman et al., 2011).

Labeo calbasu (Hamilton, 1822), an Indian major carp of the family Cyprinidae, which attains a maximum length of 90 cm (Menon, 1999), is a popular food fish and has

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potential for commercial aquaculture in Bangladesh and other South Asian countries. The fish has been reported from open waters of Bangladesh and neighboring countries including India, Pakistan, Myanmar, Thailand, and China (Talwar and Jhingran, 1991). In the past, the fish was abundantly available in all of the major rivers and floodplains of Bangladesh and currently, has been listed as an endangered fish (IUCN-Bangladesh, 2000). Development of cryopreservation protocols for this species could be an appropriate tool for ensuring genetic diversity in selective breeding programs and laboratory conservation of sperm for gene banking. A repository of frozen sperm would ensure the accessibility of a large and diverse spectrum of samples with the practical advantages of reducing dependence on synchronous maturation between sexes, facilitation of gamete transportation, and reduction of the maintenance of live broodstock (Melo and Godinho, 2006).

After collection, freshwater fish sperm must be diluted in an isotonic extender solution, which prolongs cell viability by immobilizing the sperm, and prevents death from desiccation and hypoxia (Riley et al., 2004). To develop sperm cryopreservation protocols for any species, immobilization solutions, sperm dilution ratios, activation media, cryoprotective agents, equilibration time, cooling rates, thawing rates, ionic composition and osmolality of samples should be the primary consideration (Tiersch, 2011).

Fish sperm are typically immotile in seminal plasma (Alavi et al., 2009) and therefore, diluents should be prepared with osmolality similar to that of seminal plasma. Among the different cryoprotectants, dimethyl sulfoxide (DMSO) and methanol (which quickly permeates cells) have been tested at different concentrations to establish the equilibration time necessary to protect cells from toxicity, dehydration and ice crystal formation. After equilibration, sperm samples are cooled at a range of rates (often 5–15 °C/min) in the vapor of liquid nitrogen and stored in liquid nitrogen dewars (Cabrita et al., 2005).

Sperm cryopreservation of cyprinid fish has been studied for a considerable number of species around the world and, it is possible to gain a general understanding of the broad range of conditions necessary for cryopreservation. Compared to the number of protocols developed for sperm cryopreservation of the cyprinids, very few studies have been conducted to date for the uniform cryopreservation technique. Although uniform protocol might be useful, there are chances of variation of the spermatological properties, toxicity tolerance level to cryoprotectants that can affect fertilization of eggs with cryopreserved sperm for each of the species. The study reports on the preliminary aspects of sperm cryopreservation of *L. calbasu* therefore, the basic aspects have been addressed rather considering to develop a uniform protocol. The suitability of protocols can be tested by evaluating motility of the thawed sperm. However, the ultimate criterion is the ability to fertilize eggs, and consequently good survival and growth of hatchlings (Honeyfield and Krise, 2000). Furthermore, the sperm-to-egg ratio of cryopreserved sperm needs to be optimized, otherwise, excess sperm could be used for fertilization. Accordingly, the objectives of the present research were to test the following: (1) equilibration of *L. calbasu* sperm with different cryoprotectants in graded concentrations and time; (2) different cooling and thawing temperatures with appropriate cooling and thawing rates, and (3) fertilization capacity and appropriate sperm-to-egg ratios for thawed sperm.

2. Materials and methods

2.1. Collection and rearing of broods

Broods of *L. calbasu* were collected in 2007 and 2008 from two major rivers in Bangladesh – the Jamuna (Bogra District) and the Halda (Chittagong District) and a *haor* (Netrokona District). Collected broods were transported to the rearing ponds of the Fisheries Field Laboratory Complex, Bangladesh Agricultural University (BAU), Mymensingh. In the rearing ponds, the broods were fed commercial diet (35% protein; Paragon Feeds Limited, Bangladesh) twice daily at 4–5% of the body weight of the fish.

2.2. Milt collection, dilution and activation

Milt samples were collected from *L. calbasu* during the breeding season of two successive years (May–July 2008 and 2009). Males were injected intraperitoneally with carp pituitary supernatant (2 mg kg^{-1} body weight) for induction of spermiation. Six hours after injection, gentle pressure was applied to the abdomen and milt samples were collected in plastic tubes containing 1.0 ml Alsever's solution (0.7% NaCl, 0.8% sodium citrate dissolved in 100 ml water; osmolality 296 mOsmol kg^{-1}) which had sufficient osmotic pressure to immobilize the sperm. The collected samples were placed on ice (4 °C) and brought to the Laboratory of Fish Biodiversity and Conservation for further study. Samples were diluted at 1:9 ratio based on results of prior experiments (Hassan et al., 2012) on dilution ratios of *L. calbasu* sperm. The suitability of diluents and dilution ratios were evaluated with respect to percent motility of the stored sperm after activation.

The percent motility of sperm was estimated by visual observations of two observers with three replications for each sample using a light microscope (Novex K-range, Netherland) at 400× magnification. Sperm motility was estimated by adding 10 μl of 0.3% NaCl (96 mOsmol kg^{-1}) as activating medium to 1 μl of fresh milt on a glass slide. The motility was observed within 4–6 s after activation and was expressed as the percentage of sperm that had forward movement.

2.3. Evaluation of sperm concentration

Sperm counts were performed in triplicate by haemacytometer and expressed as the number of cells per ml. Sperm was diluted 4000-fold in 0.3% NaCl, and a droplet of the diluted sperm was placed within a haemacytometer (area of the smallest square = 1/400 mm^2, depth 0.1 mm) with cover slip (Marienfeld, Germany). A total of ten minutes were allowed to sediment all the sperm. The number of spermatozoa in five large squares (area 1/25 mm^2) of the counting chamber was counted at 400× magnification.