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SHORT COMMUNICATION

Motility activation and short-term storage of silver carp, *Hypopthalmichthys molitrix*, sperm

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Abstract Sperm activation and short-term storage have important implications in breeding programs. Although silver carp, *Hypophthalmicthys molitrix*, is an important aquaculture species in South Asia, information on sperm biology and storage is limited. The study aims to test the efficacy of NaCl solutions for activation and short-term storage of silver carp sperm. We found that the motility of silver carp sperm lasted for 45 sec after activation in distilled water. Sperm motility was higher within the first 10 sec of sperm activation, but sperm movement decreased with increasing activation time. When sperm was suspended a graded dilution of NaCl solutions (17 mM to 188 mM), sperm was immobilized at 154mM NaCl (287 mOsmol kg^{-1}). The efficacy of sperm immobilizing solutions was validated during short-term storage at 4°C. A total of 7.1 ± 3.1% sperm was motile in 154 mM NaCl after 5 days of storage. Sperm motility lasted up to 2 days when sperm diluted in 103, 120, 171 and 188 mM NaCl solutions. The osmotic balance between seminal plasma and NaCl solution prepared at 154 mM prevented sperm activation and provided a suitable media for short-term storage. A 154 mM NaCl solution can be used for sperm collection and short-term storage of silver carp sperm.

Keywords Sperm motility . Short-term storage . Silver carp . *Hypopthalmichthys molitrix*

Introduction

Activation on sperm motility is vital for controlled reproduction. In most freshwater fishes, sperm remains immotile in seminal plasma, but when sperm is released to the external medium, osmolality of the medium triggers sperm motility. In addition, the differences in Na, K and Ca^{2+} ions of the external medium vs seminal plasma activate sperm motility (Alavi et al. 2009). Sperm motility is short in freshwater fish due to the difference in osmotic pressure between sperm and external medium. Contamination with water or urine during sperm collection activates sperm motility prematurely due to low osmotic pressure of water or urine, and sperm lose fertilization capacity (Perchec et al. 1995). Such a premature sperm motility activation can be prevented using an isotonic solution or seminal artificial plasma (ASP) during sperm collection (Cejko

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et al. 2019). Several media have been developed and utilized as for short-term sperm storage such as phosphate buffer, Alsever's solution, Tyrode's medium, frog Ringer's solution, modified Krebs- Ringer's, Cortland's fluid and modified Cortland's fluid (Ravinder et al. 1997; Glenn et al. 2011). Recently, ASP containing 2mM $CaCl_2$, 1mM Mg_2SO_4, 20mM Tris, 110mM NaCl and 40mM KCl used for common carp sperm storage that provided twice higher motile sperm than undiluted sperm after 72 h storage (Cejko et al. 2018). Sodium chloride can also be used as an isotonic solution to immobilize sperm motility. The advantage of sodium chloride is its wide availability and low cost. However, sodium chloride's efficacy at different concentrations must be optimized before using a sperm immobilizing solution.

Short-term storage of sperm facilitates transportation and breeding programs. This technique allows low temperatures (4°C) sperm storage for days to weeks without losing fertilizing capacity (Bobe and Labbe 2009; Shaliutina et al. 2013). Factors such as extender selection and sperm dilution ratio are important to achieve an optimal condition for sperm storage (Sarosiek et al. 2012). In addition, factors such as ions, pH, oxygen and air atmosphere also affect viability of sperm (Kowalski et al. 2014; Cejko et al. 2018). Although cryopreserved sperm can be stored long-term to preserve important fish species' gametes, cryopreservation is a high-tech and costly approach. As such, cryopreservation is not suitable for marginal scale hatchery and farm operations.

On the other hand, short-term storage of sperm is easy and cheap, but it has many applications. Fresh sperm from different locations can be stored short-term and transported for fertilization in commercial hatchery operations (Bobe and Labbe 2009; Sahin et al. 2013). Short-term storage could solve the problems of inbreeding of other native species currently held in captivity. Furthermore, short-term storage has been widely used in breeding programs of commercially important species (Contreras et al. 2020). However, a suitable isotonic media for sperm dilution during short-term storage is required, which can be made using salt solutions.

Silver carp, *Hypohthalmichthys molitrix*, is a freshwater cyprinid fish species that was introduced in Bangladesh from Hong Kong. Faster growth rate and this species' ability to live harmoniously with other species, silver carp has become an attractive option for polyculture in Bangladesh, with a total 6.7% contribution to the total inland aquaculture production in Bangladesh (DoF 2018). Currently, information on sperm motility activation and short-term storage of silver carp sperm is lacking. This study aims to test the efficacy of NaCl solutions in short-term storage of silver cap sperm.

Materials and methods

Sperm collection

Brood fishes (length 43.2 ± 2.2 cm, weight 2.2 ± 0.2 kg) from a commercial fish hatchery were used in this study (High Fish Hatchery, Dinajpur, Bangladesh 25°47'50.9"N 88°34'53.4"E). Matured males were caught from the broodstock pond and transferred to the cistern before the day of sperm collection. This study included a total of 28 males. Selected broods were injected intramuscularly with one dose of pituitary gland extract (commercially available dry PG) at 2mg/kg. Injected fishes were maintained at 26 °C for 6-8 h with a continuous water shower. The fish were then removed from the cistern, and the genital area was thoroughly cleaned of any water, urine, or mucus. Gentle pressure was applied on the abdomen, and initial sperm was wiped off to avoid contamination with urine and water. Sperm was collected in 1.5 ml eppendorf tube, and initial sperm motility from each brood was evaluated under the light microscope. Sperm motility was evaluated by subjective method, based on evaluations of two independent observers. Aliquots of sperm with at least 80% motility were used for this study.

Sperm motility activation solution

A series of NaCl solutions (17 to 188 mM) prepared at osmolality 48-351 mOsmol kg^{-1} was used to activate sperm. Based on the activation curve, 103 to 188 mM NaCl solutions were evaluated to test their efficacy in the short-term storage of sperm. Sperm were diluted in NaCl solutions at 1:5 ratio and packaged in 1.5 ml eppendrof tubes and stored in the refrigerator for all experiments.

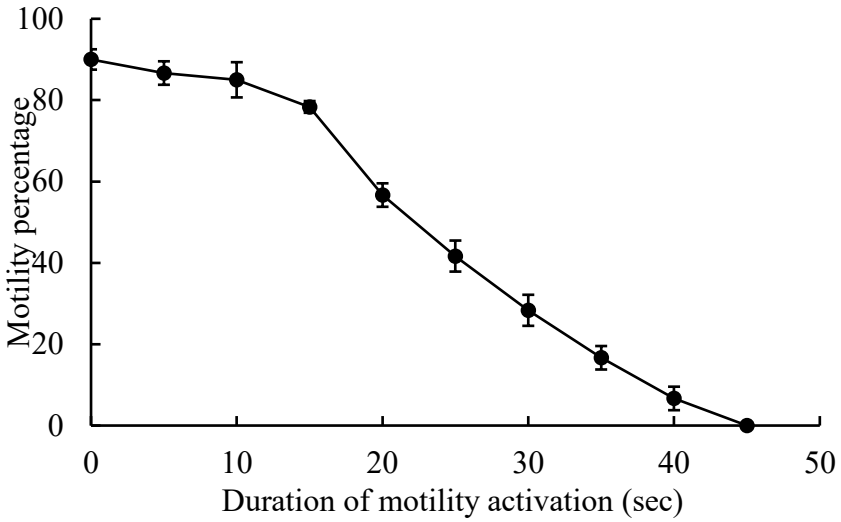

Fig. 1 Motility of silver carp *Hypopthalmichthys molitrix* sperm activated with distilled water. Aliquots of sperm from three males were used in each observation, and each point represents the mean ± standard deviation of three observations.

Motility evaluation

A light microscope at 40x objective lens was used to visualize sperm motility. During motility evaluation, 1 µl of sperm solution is added to pre-loaded 19 µl distilled water for all observations. During sperm motility evaluation, samples were retrieved from the refrigerator and warmed up in room temperature for 2 min before activation of motility in distilled water. The motility duration was counted from the time of activation to cessation of forward movement. The motility of stored sperm was assessed twice a day for up to 5 days.

Statistical analysis

Prior to data analysis, assumptions of normality and homogeneity of variances were tested by Shapiro–Wilk and Levene's test statistic. The effects of sodium chloride solutions on storage durations were analyzed using a one-way analysis of variance (ANOVA). Tukey post-hoc test was applied when a significant difference ($P < 0.05$) was found among the response variables. All the data were analyzed using SPSS v. 27 (IBM, Armonk, NY).

Results

Motility characteristics

When sperm motility was activated using distilled water, the motility of sperm lasted for 45 seconds. However, the motility percentage decreased with increasing activation duration. In the first 10 seconds of activation in distilled water, over 80% of sperm showed forward movement (Fig. 1).

Activation of sperm motility at different osmolality

When sperm motility was activated using NaCl solutions' different osmolality, the highest motility precentage was observed at 48 mOsmol kg^{-1} (17 mM NaCl solution). Sperm motility completely immobilized at 287 mOsmol kg^{-1} (154 mM NaCl solution). The motility percentage of sperm decreased with increasing osmolality from 48 to 351 mOsmol kg^{-1} (Fig. 2).

Short-term storage of sperm

The initial motility of sperm before storage was 89.1 ± 3.7%. Motility percentage of sperm declined sharply

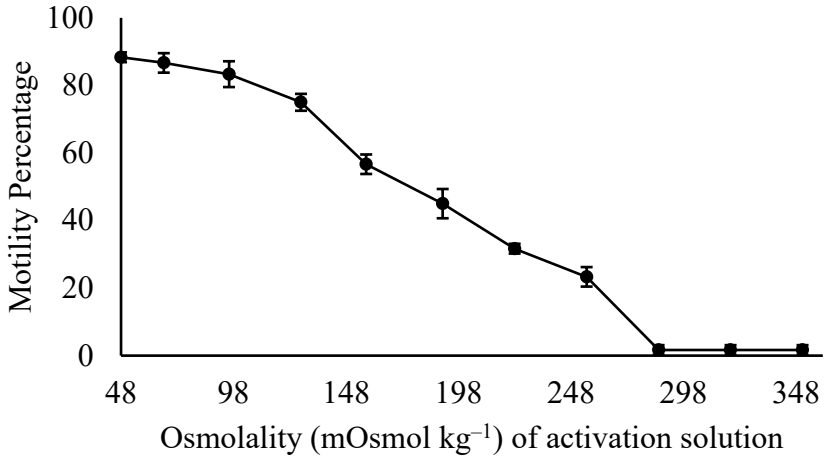

Fig. 2 Motility of silver carp *Hypopthalmichthys molitrix* sperm exposed to NaCl solutions prepared at graded osmolality. Aliquots of sperm from three males were used in each observation, and each point represents the mean ± standard deviation of three observations.

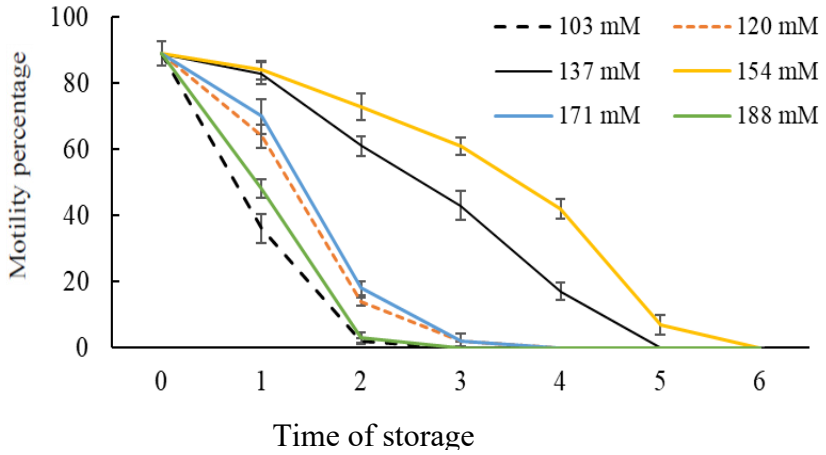

Fig. 3 Motility percentage of silver carp *Hypopthalmichthys molitrix* at different storage time when suspended at graded concentrations of NaCl solutions (nM). Sperm from three males are pooled in each observation and each point represents the mean of three batches of sperm.

after two days of storage when diluted in mM, 120 mM, 171 mM and 188 mM NaCl solutions. However, 7.1 ± 3.1% of sperm were found motile in 154 mM NaCl after 5 days of storage (Fig. 3).

During short-term storage, the highest storage time was found when sperm were suspended in 154 mM NaCl. Although the storage time of sperm was lower in 137 mM NaCl, this treatment is not significantly different from 154 mM NaCl. The storage time in other treatments such as 103 mM, 120mM, 171 mM, and 188mM NaCl was significantly different (Fig. 4).

Discussion

This study indicates that the silver carp sperm motility is very short, and sperm should be in contact with eggs shortly after motility activation. Besides, 154 mM NaCl solutions can be used to immobilize and short-term storage of silver carp sperm.

Duration of sperm motility is controlled by osmotic gradient and sperm energetics. The osmolality of distilled water is very low compared to the seminal plasma of fish; thereby, sperm movement lasts for seconds to minutes when freshwater fish sperm is exposed to distilled water. When sperm gets activated with distilled water, the velocity of sperm movement also decreases sharply after activation (Perchec et al. 1996). Comparing different activation mediums for common carp sperm using CASA, distilled water activation rendered the lowest sperm motility parameters such as progressive motility, movement velocity,

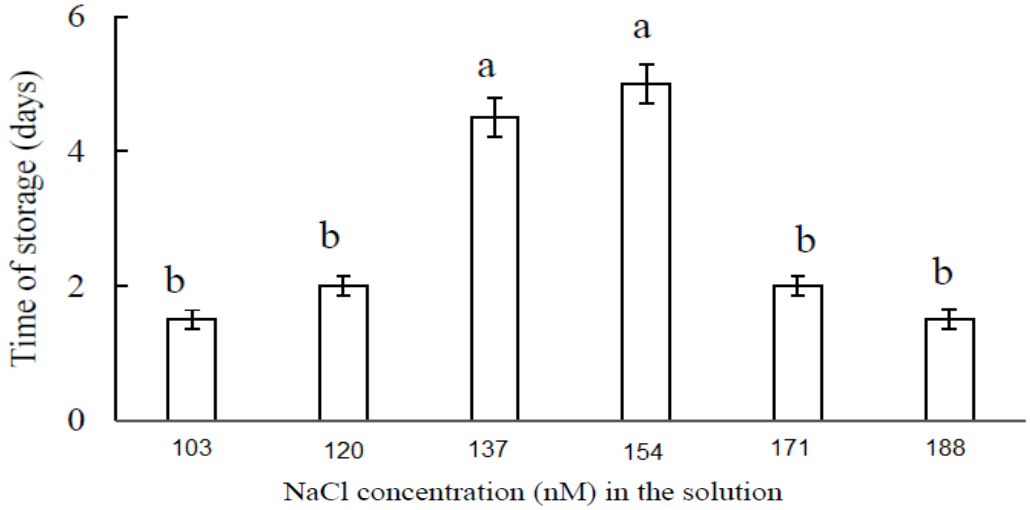

Fig. 4 Storage duration of silver carp *Hypopthalmichthys molitrix* sperm suspended at graded concentrations of NaCl solutions. Boxes marked with different letters indicate a significant effect of NaCl concentration on storage time ($P < 0.05$). Sperm from three males are pooled in each observation, and each point represents the mean of three batches of sperm.

and beat cross frequency (Cejko et al. 2013). In this study, the sperm movement was high in the initial 10 sec of activation, which gradually decreased over time and lasted for 45 seconds. A similar duration of sperm movement was observed in other freshwater species, including *Puntius sarana* - 35 sec (Nahiduzzaman et al. 2011), *Sander vitreus* - 51 sec (Satterfield and Flickinger 1995) and *Ptychocheilus lucius* - 57 sec (Tiersch et al. 2004), *Labeo calbasu* - 60 sec (Hassan et al. 2013). Besides, it has also been reported that the major ions involved in improving motility characteristics is Na^+ (Khara et al. 2012). A higher sperm movement duration was also reported in some other freshwater fishes, such as 4-5 min in *Polyodon spathula* (Mims 1991), 6-7 min in *Esox masquinongy* (Lin and Dabrowski 1996). Short duration of sperm movement in silver carp has important implications in controlled breeding - sperm should encounter eggs shortly after activation to achieve a higher fertilization rate. Although faster mixing of sperm with egg would increase the rate of fertilization success, care should also be taken for gentle mixing. Otherwise, delicate sperm tail and egg membrane could be damaged by rough handling.

Even though NaCl solutions were used for motility activation in this study, NaCl solution may not offer optimal conditions for cyprinid sperm activation. In previous studies, cyprinid sperm activated using Woynarovich solution (68 mM NaCl and 50 mM urea, pH 7.7 and osmolality 180 mOsm/kg) provided higher motility sperm than other activation media (Cejko et al. 2013; Kowalski and Cejko 2019). Buffer solutions containing 5 mM KCl, 45 mM NaCl, and 30 mM Tris was also effective in carp sperm activation (Saad and Billard 1987). The efficacy of other activation media is worth of testing for silver carp sperm to exploit better outcome in sperm motility activation.

In this study, sperm motility was evaluated by a subjective method which is limited by human error. The computer assisted sperm analysis (CASA) offers an objective estimation of sperm motility without any human error (Boryshpolets et al. 2013; Kowalski and Cejko 2019). However, subjective measure of sperm motility is widely used in assessing sperm quality during egg fertilization (Kucharczyk et al. 2020). Subjective estimation of sperm motility has wider applicability in fish hatcheries and field sampling. The subjective method also offers a reliable estimation of sperm motility when motility estimation is done by an experienced technician (Gallego et al. 2018).

The osmolality of seminal plasma in freshwater fish ranges from 230-346 mOsmol kg^{-1} (Alavi and Cosson 2006). Suspension of sperm in an iso-osmotic solution prevents sperm activation. In this study, sperm was activated using NaCl solutions prepared at 46-351 mOsmol kg^{-1}. Sperm of silver carp was immobilized when suspended at 287 mOsmol kg^{-1}. In other freshwater fishes, sperm was immobilized using osmolality 296 mOsmol kg^{-1} of Alsever's solution for *Puntius sarana* (Nahiduzzaman et al. 2011) and 287 mOsmol kg^{-1} of NaCl solutions for *Labeo calbasu* (Hassan et al. 2013). Implications of sperm immobilizing solutions of silver carp sperm include short and long-term storage using this solution. Furthermore, this

solution can be used to collect sperm to reduce sperm activation caused by contamination or mixing with water and urine. Furthermore, such a solution can also be used for sperm transportation from one farm/hatchery to another.

The efficacy of NaCl solutions was further tested for sperm storage. Among the NaCl solutions concentrations were tested, the highest storage duration was achieved using 154 nM NaCl. This was due to the conformity of the osmotic balance between NaCl solution and seminal plasma. A similar storage duration was also achieved using 137 nm NaCl. However, higher or lower concentrations of NaCl solutions were unsuitable for silver carp sperm storage. Similar to this species, *Labeo calbasu* sperm were stored up to 72 hrs using 0.9% NaCl (~ 154 nM) solutions (Hassan et al. 2013). Relatively longer storage duration of 19 days was achieved in Koi carp using Calcium free Hanks' balanced salt solution prepared at 270 mOsmol kg^{-1} (Glenn et al. 2011).

Conclusion

Information presented in this study on sperm activation and short-term storage would improve gamete collection, transportation, and breeding programs. This technique is cost-effective and can be adopted in small and medium-scale fish hatcheries in South Asia. This information could also be applied in selecting suitable media for cryopreservation of silver carp sperm.

Declaration of competing interest: The authors declare that they have no conflict of interest.

Author's contributions: CR - data acquisition and prepare original draft, IH - data acquisition and manuscript revision, MAM - data acquisition and prepare original draft, IP – conceptualization and project management, NWR - data interpretation and manuscript revision, MMH – conceptualization, data analysis, data interpretation and manuscript revision.

Compliance with ethical standards: The institutional guidelines for use of animals in research were followed by the authors.

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