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***Research Article** (Open Access)*

EFFECTS OF CRYOPRESERVATION ON MOTILITY, VIABILITY, ULTRASTRUCTURE, FUNCTIONAL INTEGRITY AND DNA INTEGRITY OF SPERMATOZOA IN INDIAN SAND WHITING *SILLAGO SIHAMA* (FORSSKAL, 1775)

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ABSTRACT
Cryopreservation involves preserving live cells and tissues in a structurally stable and intact condition for longer duration at a fairly low cost at very low temperatures. The worldwide increase in aquaculture production through intensive systems has emphasized the need for developing effective protocols for conserving gametes. Preserving gamete ensures flexibility in brood stock management, genetic improvement programs as well to conserve genetic diversity. Indian Sand Whiting, *Sillago sihama* is an ideal candidate for aquaculture. An attempt has been made to study the effects of cryopreservation on sperm motility, viability, ultrastructure, functional integrity & DNA integrity in Indian Sand Whiting *Sillago sihama*. Cryopreservation of milt was carried out using cryomedium constituting Marine Ringer (Burton, 1988) with 10% DMSO (Equilibration time of 10 minutes). Cryoprotectants were assessed at different concentrations individually as well as in combination with varying equilibration time. The samples were plunged in liquid nitrogen (−196 °C) and stored for a period of 30 days. Samples were subsequently thawed in a water bath at 30 °C for assessment of sperm motility, viability, ultrastructure, functional integrity & DNA integrity. The present study, therefore, provide base-line data for future experiments.

Keywords: *Cryopreservation; Sperm; Damage; Sillago sihama*

INTRODUCTION
The Sand Whitings of family Sillagnidae are well accepted as food fish around the world. Sillaginidae has conventionally been an important fish family valued in many countries throughout the world. Despite being an important aquaculture species, studies related to cryopreservation of spermatozoa of *Sillago sihma* are limited. Cryopreservation involves preserving live cells and tissues in a structurally stable and intact condition for longer duration at a fairly low cost at very low temperatures. It can ensure constant supply and availability of gametes, conserving of endangered and threatened species well as improving the existing to genetic resources (Tiersch, Yang, Jenkins & Dong 2007; Tiersch 2011; Tiersch, Yang & Hu 2012; Viveiros et al.,2012). The foremost studies on cryopreservation of fish sperm was by Blaxter, 1953, and more than 200 fish species have been studied (Billard & Zhang 2001; Gwo 2011). About 40 of it are marine species (Suquet *et al.*, 2000; Gwo 2011). This reliable method can be used for storing a wide range of cells and some tissues. Although, successful sperm cryopreservation has been reported in some species, no technique has been developed for the routine sperm cryopreservation for all fish species. To the best of our knowledge, there has been no study to document the effects of cryopreservation on motility, viability, ultrastructure, functional integrity and DNA integrity in *Sillago sihama*. Hence, the present study was conducted to evaluate the changes in cryopreserved the spermatozoa of *Sillago sihama*

MATERIALS AND METHODS
Milt Collection
Live male specimens (n=10) of *Sillago sihama* for the study were collected from the Vypin and Fort Kochi by Chinese dip net operations from the local fishermen.

Milt was collected from the live fishes after cleaning the genital opening with dry sterile cotton. Milt from the fishes were pooled stripped into 2 mL centrifuge tubes and kept above crushed ice in insulated ice box, transported to the laboratory and stored at 4 °C until further use. Care was taken to avoid contamination of milt with urine, faeces, blood or mucus. The stored milt was in such a way the samples are not in contact with water. Contaminated samples were discarded.

Assessment of sperm quality

Spermatozoa Motility

For sperm motility assessment, 5µL of fresh sperm was placed on a clean microscopic slide and diluted with 30µL of filtered natural seawater (pH 8.1 and salinity 32 ppt) collected from the sampling site. The motility grade of each sperm sample was assessed under under a bright field microscope (Biolinkz).

Motility score was assigned based on Goodall *et al.*, (1989) with slight modification.

Scoring 0 100% Spermatozoa immotile, I <30% Spermatozoa actively motile, II 30-50% Spermatozoa actively motile, III 50-70% Spermatozoa actively motile, IV 70-80% Spermatozoa actively motile, V >80% Spermatozoa actively motile.

For all experiments, freshly collected sperm with the motility grade of 4 was used.

Spermatozoa Viability

Sperm viability was evaluated using the eosin-nigrosin staining method (Zaneveld and Polakoski, 1977). For viability assessment, a drop of milt was mixed with one drop of 0.5% eosin (aqueous solution) and two drops of 10% nigrosin (aqueous solution) on a clean slide. A thin uniform smear was prepared and air-dried. The slides were observed under light microscope within 2 min of smear preparation. Live sperm cells appeared unstained (grey) whereas dead sperm cells were pink.

Spermatozoa Morphology- Scanning Electron Microscopy

The sperm samples were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer at 4 °C, after which the samples were postfixed and washed repeatedly in 4% osmium tetroxide and dehydrated through a acetone series (Hatef *et al.,* 2011). The samples were air dried and coated with gold in a sputter coater and observed under a scanning electron microscope (JEOL Model JSM - 6390LV) and electron micrographs were obtained. Scanning Electron micrographs of fresh as well as cryopreserved spermatozoa was obtained

Cryopreservation Protocol

The milt samples were diluted (1:3 v/v milt +cryomedia (Extender + cryoprotectant)) prepared using Marine Ringer (Burton, 1988) + 10% DMSO with equilibration time 10 minutes. In our earlier studies the extender, dilution ratio, cryoprotectant concentration, equilibration time and freezing rate have been standardized for *Sillago sihama* (Revathy, 2019). They were loaded into 1.5 ml cryovials and were then placed in canisters. The canisters were clamped 2 cm above the liquid nitrogen in the vapour phase in the cryocan for 5 minutes and then the canisters with the vials immediately plunged into liquid nitrogen at -196°C for storage. The cryopreserved samples were thawed after 30 days at 30°C for 5 min in a water bath and analyzed for sperm quality motility, viability, functional integrity, DNA integrity and Scanning Electron Microscopy.

DNA Integrity Assessment using Acridine Orange Staining Assay

DNA integrity of cryopreserved samples after 30 days were analysed using the acridine orange staining method by Varela Junior et al. (2012).

Functional Integrity Assessment with Hypo-Osmotic Swelling Test

For functional integrity assessment, 0.1 ml of undiluted semen and 1.0 ml of hypo-osmotic solution was mixed and incubated at 4 °C for 15 min. A drop of diluted semen was placed on a clean dry glass slide, stained with eosin and nigrosin and covered with a cover slip and observed different fields at phase contrast (Leica DM 2000 Germany) and bright field (Biolinkz). Positive HOS test was indicated by Presence of coiled tailed in spermatozoa. The percentage of spermatozoa positive to HOS test was determined using standard protocol (Jeyendran *et al.,* 1984).

Statistical analysis

All data are expressed as mean ± SD. Statistical package (SPSS) 23.0 USA was used for the statistical analysis.

RESULTS

Viability & Motility

Freshly collected uncontaminated milt was white in colour and mucilaginous in nature. Viability is the measure of the physiological functional aspect of spermatozoa and percentage of motile spermatozoa indicates the number of spermatozoa, which can actively move in the medium. Viability of spermatozoa of *Sillago sihama* was 89.8 ± 4.20 % and percentage of motile spermatozoa was 86.2 ± 2.77 % (Fig. 1.). The Duration of motility is the time up to which spermatozoa can remain motile in a particular medium. The duration of motility of sperm of *Sillago sihama* was 91.2 ± 3.19 (seconds).

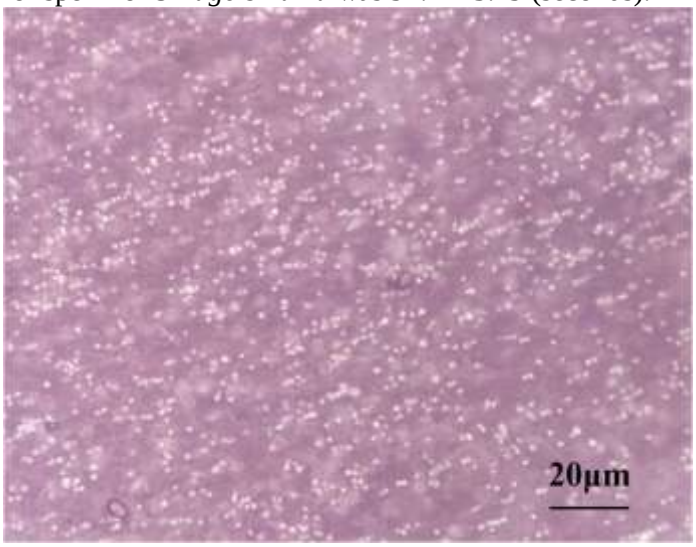

Figure. 1. Sperm Viability using Eosin Nigrosine Staining
**Viable sperm exclude the stain; While Non Viable sperm include the stain*

Spermatozoa Morphology- Scanning Electron Microscopy

The spermatozoa of *Sillago sihama* had characteristics of typical fish spermatozoa with a uniflagellated cell, with a prominent head, mid-piece and long cylindrical flagellum. Acrosome structure was absent. Head was elongated spherical with 1.81 ± 0.05 µm in length anterio-posteriorily and a diameter 1.38 ± 0.03 µm. The mid-piece measured about 0.25± 0.02 µm in length. The tail originated from conical mid-piece and had a length of 14.50 ± 0.03 µm. The plasma membrane covering the head and tail was smooth in appearance. The orientation of mid piece and tail supported the bilaterally symmetrical appearance of spermatozoa (Fig. 2.).

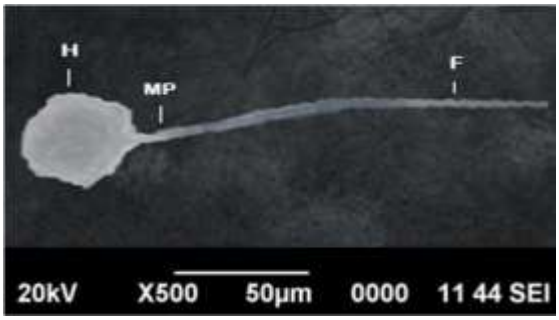

Figure 2: Scanning electron micrograph of spermatozoa of *Sillago sihama*

Quality of post-thaw cryopreserved sperm after 30 Days of Storage in liquid nitrogen at -196°C

Milt diluted with Marine Ringer (Burton, 1988) and DMSO (10 %) showed maximum motility of 59.00 ± 1.73% and viability of 63.67 ± 1.15 %. Functional integrity and DNA integrity were also assessed. On 30 days of storage in Marine Ringer (Burton, 1988) and DMSO (10 %), the spermatozoa of *Sillago sihama* exhibited functional integrity of 59.33 ± 4.73 and DNA integrity of 73.67 ± 5.69. At 30 days of storage, the structure of head of spermatozoa was more or less intact and flagellum showed minimal bulging (Figs. 3, 4, 5, 6 and 7).

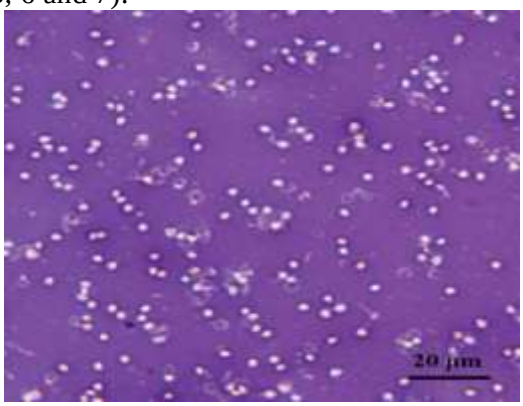

Figure 3: Control sperm exhibiting maximum tail swelling as an index of Functional Integrity

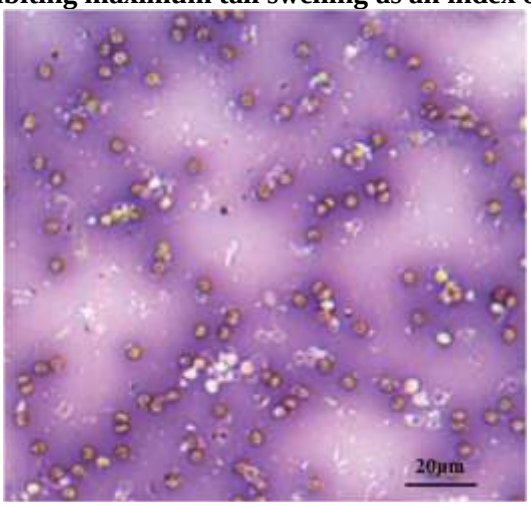

Figure 4: Functional Integrity after 30 days of storage

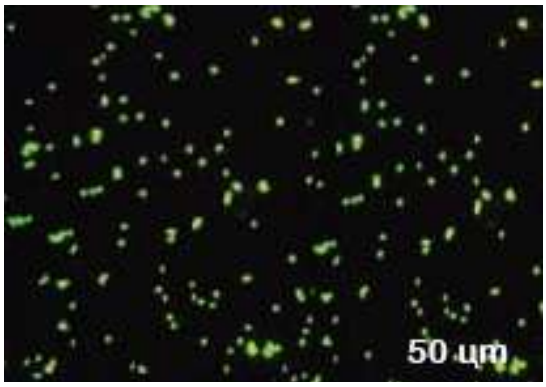

Figure 5: Control sperm exhibiting DNA Integrity

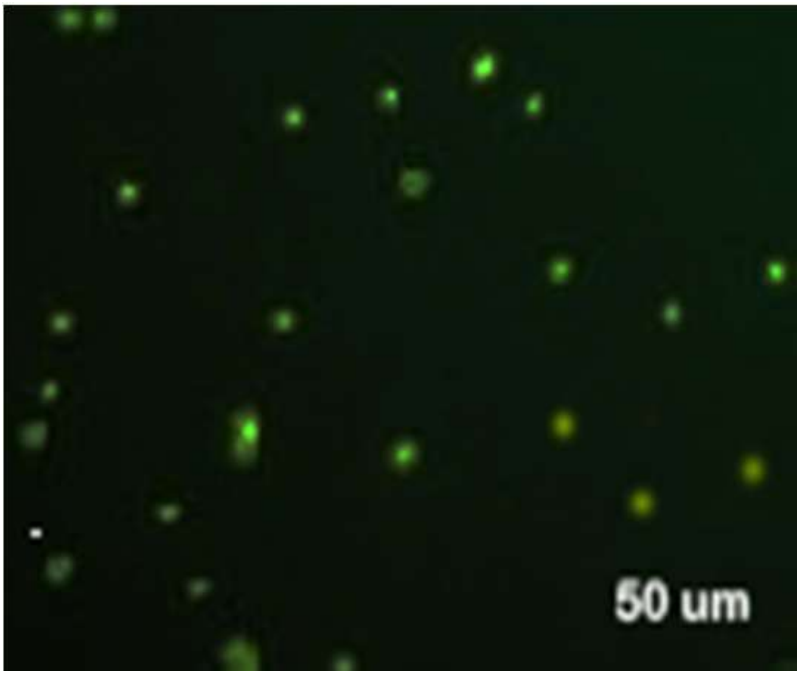

Figure 6: DNA Integrity after 30 days of storage
Green fluorescence indicates undamaged DNA; red and orange indicates damaged DNA

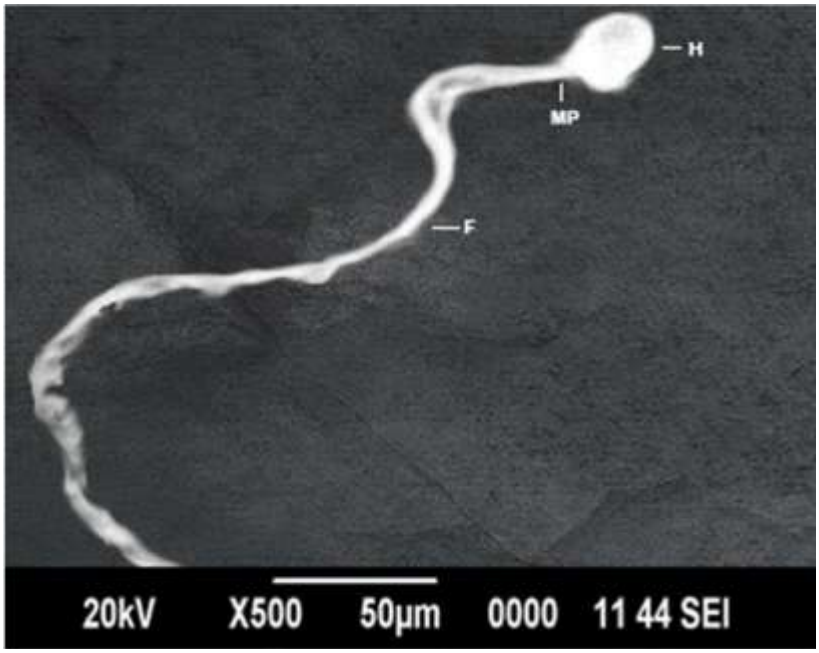

Figure 7: Scanning Electron Micrograph of cryopreserved sperm after 30 days of storage

DISCUSSION

An assessment of motility and viability of milt of *Sillago sihama* was carried out in the present study and further, an attempt has been made to develop a cryopreservation protocol. Freshly collected uncontaminated milt of *Sillago sihama* recorded a motility of 86.2 ± 2.77 %, viability of 89.8 ± 4.20 % whereas after 30 days of storage the motility of cryopreserved spermatozoa was 59.00 ± 1.73% and

viability was 63.67 ± 1.15 %. The percentage of motile spermatozoa actually corresponds to sperm viability (Viveiros *et al.*, 2012).

The generation of reactive oxygen species (ROS) during freezing- thawing process is results in oxidative stress which affects the motility, membrane stability, sperm functionality, and DNA integrity (Cabrita *et al.*, 2011; Figueroa *et al.*, 2017).

The fresh samples of milt exhibited a functional integrity of 85.67 ± 3.06 and DNA integrity of 98.33 ± 0.58. As storage days progressed the functional integrity and DNA integrity decreased. On 30 days of storage in Marine Ringer (Burton, 1988) and DMSO (10 %), the spermatozoa of *Sillago sihama* recorded a functional integrity of 59.33 ± 4.73 and DNA integrity of 73.67 ± 5.69. Lechniak *et al.*, 2002 remarked that structural and functional integrity of sperm are reliable measure that corresponds to sperm viability. While HOS test forms a much assuring method for evaluating the fertilizability of sperm plasma membrane (Madeja *et al.*, 2003). The present study also indicated a significant relation between HOST coiling, acridine orange assay, motility and viability. The DNA integrity value at 30 days DNA integrity was not compromised much after freezing- thawing. Similar results were noted in Atlantic croaker, where the freezing-thawing did not affect the DNA integrity (Gwo and Arnold, 1992). However, in other marine fishes like seabass and sea bream, a higher level of DNA fragmentation was reported with cryopreserved samples (Zilli *et al.*, 2003; Cabrita *et al.*, 2005). The differences among species are attributed to species specific resistance to cryoinjury, particular chromatin structure and the extenders used or habitat difference of the fishes (Perez-Cerezales *et al.*, 2009; Figueroa *et al.*, 2013; 2016).

Spermatozoa structure and morphology varies greatly in different fish species (Ginsburg, 1968; Turdakov, 1972; Baccetti, 1984; Jamieson, 1991; Hara and Okiyama, 1998). Scanning Electron Microscopy revealed that the spermatozoa of *Sillago sihama* had characteristics of a typical fish sperm with a uniflagellated cell, with a prominent head, mid-piece and long cylindrical flagellum. Acrosome structure was absent in *Sillago sihama*. Acrosome structure was absent in many fish species with external fertilization (Koening *et al.*, 1978 and Lahnsteiner *et al.*, 1995). Head was elongated spherical with 1.81 ± 0.05 µm in length anterio-posteriorily and a diameter of 1.38 ± 0.03 µm. Elongated head is an advanced feature than spherical head (Jamieson, 1991). In most of the teleost fishes with external fertilization, the sperm head is spherical or ovoid with around 5 µm in diameter and a small middle piece (Lahnsteiner and Patzner, 2008).

At 30 days of storage, the structure of head of spermatozoa was more or less intact and flagellum showed minimal bulging. The present study are also in agreement with the findings of Chew *et al.*, 2010; Lichtenstein *et al.*, 2010; Cabrita *et al.*, 2011 and Dogu, 2012 indicating high sperm motility and fertilization in frozen- thawed using DMSO as the cryoprotectant.

CONCLUSION

To the best of our knowledge, this is the first study that documenting the effects of cryopreservation on sperm motility, viability, ultrastructure, functional integrity & DNA integrity in Indian Sand Whiting *Sillago sihama*. The results would be helpful in optimizing the important factors to be looked into while cryopreserving spermatozoa in order minimize the cryodamage for further experiments in the future.

Conflicts of interest

None of the authors have any conflicts of interest.

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