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Effect of cryopreservation on motility, viability, functional integrity and DNA integrity of spermatozoa of Indian Sand Whiting *Sillago sihama* (Forsskal, 1775)

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ABSTRACT

Cryopreservation is a long-term storage technique at very low temperatures to preserve the live cells & tissues in a structurally stable condition for longer duration at a fairly low cost. The universal increase in aquaculture production through intensive systems has stressed the need for an efficient & effective means of conserving gametes for greater flexibility in brood stock management, genetic improvement programs & preserving genetic diversity. Cryopreservation of milt of *Sillago sihama* was studied using two different extenders, Alsever's Solution & Marine Ringer (Burton, 1988) and cryoprotectants Dimethylsulfoxide (DMSO), Glycerol (GLY), and Methanol (MeOH) at different concentrations with varying equilibration time. The samples were plunged into 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 and viability at intervals of 15 days and 30 days. For the best extender cryoprotectant combination, functional integrity & DNA integrity of spermatozoa were also assessed. Results indicated that cryomedium constituting Marine Ringer (Burton, 1988) with 10% DMSO (Equilibration time of 10 minutes) exhibited maximum motility & viability, followed by Alsever's Solution with 10 % DMSO (Equilibration time of 10 minutes). Marine Ringer (Burton, 1988) and DMSO (10 %) showed maximum functional & DNA integrity after 15 and 30 days of storage. The present study, therefore, provides baseline data for future experiments.

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Cryopreservation; Marine; Fish; Sperm; *Sillago sihama*

1. Introduction

The Sand Whitings or Sand Borers of the family Sillagnidae are immensely esteemed food fish around the world. Sillaginidae has traditionally been an important fish family as a food resource in many Indo-West Pacific countries. *Sillago sihama* is an important species, which has prospective for domestication and culture. It is a highly esteemed food fish in Kerala, a highly valued fish in Cochin, with very high demand in the local markets. Cryopreservation is a long-term storage technique with very low temperatures to preserve structurally intact living cells and tissues for an extended period of time at a relatively low cost. Cryopreservation is to preserve and store viable biological samples in a frozen state over extended periods of time. This reliable method can be used for preserving a wide range of cells and some tissues. These methods have found widespread applications in biology, biomedical technology and conservation. In recent years, with the rapid development of marine fish aquaculture, some experiments on cryopreservation have also been conducted on marine fish species of great commercial value such as turbot (Dre´anno et al., 1997; Chen et al., 2004), flounder (Richardson et al., 1999; Zhang et al., 2003), and halibut (Bolla et al., 1987; Billard et al., 1993). Although successful sperm cryopreservation has been reported in some species, no technique has been developed for routine sperm cryopreservation for all fish species. To the best of our knowledge, there has been no study where the effects of different cryoprotectants and freezing rates have been studied to achieve to develop optimal-frozen-thawed sperm quality in *Sillago sihama*. Hence, the present study was conducted to cryopreserve the sperm through different experiments by varying the combinations of extenders, various cryoprotectants at different concentrations, dilution ratio and equilibration time at intervals of 15 days and 30 days.

2. Materials and Methods

2.1. Species of Interest

Live specimens of *Sillago sihama* for the study were collected from the Vypin and Fort Kochi by Chinese dipnet operations from the local fishermen.

2.2. Milt Collection

Milt was collected from the live fish after cleaning the genital opening with dry sterile cotton. A gentle pressure was applied initially on the posterior part of the abdomen to get rid of faeces and urine. Thereafter, pressure was applied on both sides simultaneously in the posterior direction. Milt was stripped into 10 ml polypropylene storage vials and kept above crushed ice in insulated ice box. Care was taken to avoid

contamination of milt with urine, faeces, blood or mucus and also the stored milt was in no way allowed to come in contact with water. Contaminated samples were discarded.

2.3. Assessment of sperm quality

2.3.1. Spermatozoa Motility

The motility of spermatozoa was assessed after the addition of 30 µl filtered; UV irradiated natural seawater (pH 8.1 and salinity 32 ppt) with 5µl of milt. The motility of spermatozoa was observed under a bright field microscope (Biolinkz). Duration of motility was recorded

from the time of mixing of milt with sea water to the time up to which at least 20% of spermatozoa showed active forward movement. The percentage of motile spermatozoa was calculated.

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.

2.3.2. Spermatozoa Viability

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

2.4. Cryopreservation Protocol

Based on the preliminary evaluation, the best equilibration time-concentration for each extender-cryoprotectant combination was found and following 6 combinations of cryoprotectant

Alsever's + 10% DMSO with equilibration time 10 minutes

Alsever's + 15% Glycerol with equilibration time 15 minutes

Alsever's + 15% Methanol with equilibration time 10 minutes

Marine Ringer (Burton, 1988) + 10% DMSO with equilibration time 10 minutes

Marine Ringer (Burton, 1988) + 15% Glycerol with equilibration time 15 minutes

Marine Ringer (Burton, 1988) + 15% Methanol with equilibration time 10 minutes

The milt samples were diluted (1:3 v/v milt +cryomedia (Extender + cryoprotectant)) and maintained at 4 °C for a definite equilibration time. This process was done for all the cryomedia and equilibration combinations mentioned above. 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 15 days and 30 days at 30°C for 5 min in a water bath and analyzed for sperm quality (motility and viability).

2.5. DNA Integrity Assessment using Acridine Orange Staining Assay

The acridine orange staining method by Varela Junior et al. (2012) was used to assess the sperm DNA integrity for the cryopreserved samples. The assay was limited only to the cryopreserved sample with the highest motility and viability on post-thaw analysis after 15 days and 30 days.

2.6. Functional Integrity Assessment with Hypo-Osmotic Swelling Test

0.1 ml of undiluted semen and 1.0 ml of hypo-osmotic solution were 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). The presence of coiled tails in spermatozoa indicated a positive HOS test. The percentage of spermatozoa positive for the HOS test was also determined (Jeyendran et al., 1984). The assay was limited only to the cryopreserved sample with the highest motility and viability on post-thaw analysis after 15 days and 30 days.

2.7. Statistical analysis

Statistical package (SPSS) 23.0 USA was used for the one-way Analysis of variance (ANOVA) followed by a post-hoc test (Duncan's multiple range test) to determine the level of significance ($P<0.05$) for the data obtained.

3. Results

3.1. Viability & Motility

Freshly collected uncontaminated milt was white in colour and mucilaginous in nature. Viability indicates the physiologically functional spermatozoa and percentage of motile spermatozoa denotes the number of spermatozoa, that can actively move in the medium. The viability of spermatozoa of *Sillago sihama* was 89.8 ± 4.20 % and the percentage of motile spermatozoa was 86.2 ± 2.77 % (Fig. 1). Duration of motility is the time up to which spermatozoa remain motile in a particular medium. The duration of motility of sperm of *Sillago sihama* was 91.2 ± 3.19 (seconds). Motility score is also another way of expressing motility and is represented as grades I- V. Since *Sillago sihama* had a high percentage of motile spermatozoa, a score of V was given.

3.2. Cryopreservation Protocol

The best cryoprotectant concentration-equilibration time pair of each extender and cryoprotectant combination (six combinations) was shortlisted based on cryoprotectant toxicity studies and non-frozen storage studies at 4^0C. Marine Ringer (Burton, 1988) with 10 % DMSO at an

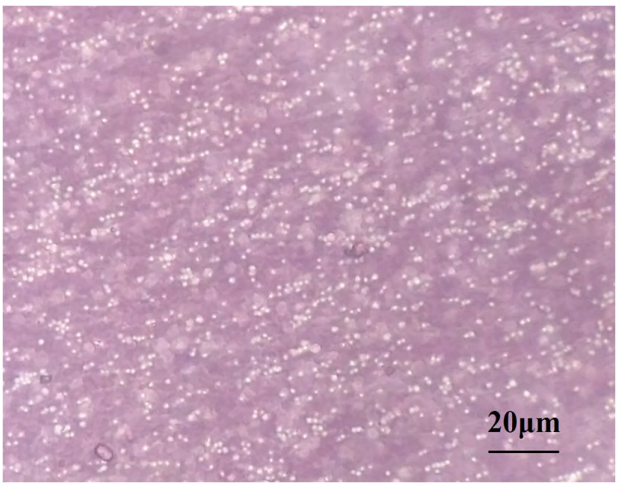

Fig. 1. Sperm viability using Eosin-Nigrosine Staining

equilibration time of 10 minutes showed higher motility (84.67±2.52 %) and viability of (90.00 ± 3.00 %), followed by Alsever's Solution with 10 % DMSO at an equilibration time of 10 minutes with a motility and viability of 82.67 ±2.52 % and 89.00 ± 3.00 % respectively. Marine Ringer (Burton, 1988) with 15 % Glycerol at an equilibration time of 15 minutes showed motility (81.67 ± 2.52 %) and viability of (85.62 ± 2.52 %) Alsever's Solution with 15 % Glycerol at an equilibration time of 15 minutes recorded motility (80.67 ± 2.52 %) and viability (84.67 ± 2.52 %). Marine Ringer (Burton, 1988) with 15 % Methanol at an equilibration time of 10 minutes motility (68.00 ± 2.00 %) and viability of (71.67 ± 2.52 %) and Alsever's Solution with 15 % Methanol at an equilibration time of 10 minutes also had considerable motility of (65.00 ± 2.00 %) and viability of (70.67 ± 2.52 %). Beyond 15 min of equilibration time, there was a deterioration in sperm quality (Fig. 2 & 3).

3.3. A Quality of post-thaw cryopreserved sperm after 15 Days of Storage in liquid nitrogen at -196°C

Milt diluted with Marine Ringer (Burton, 1988) and DMSO (10 %) showed maximum motility of 73.67 ± 2.52% and viability of 78.67 ± 2.08 %. Alsever's Solution with DMSO

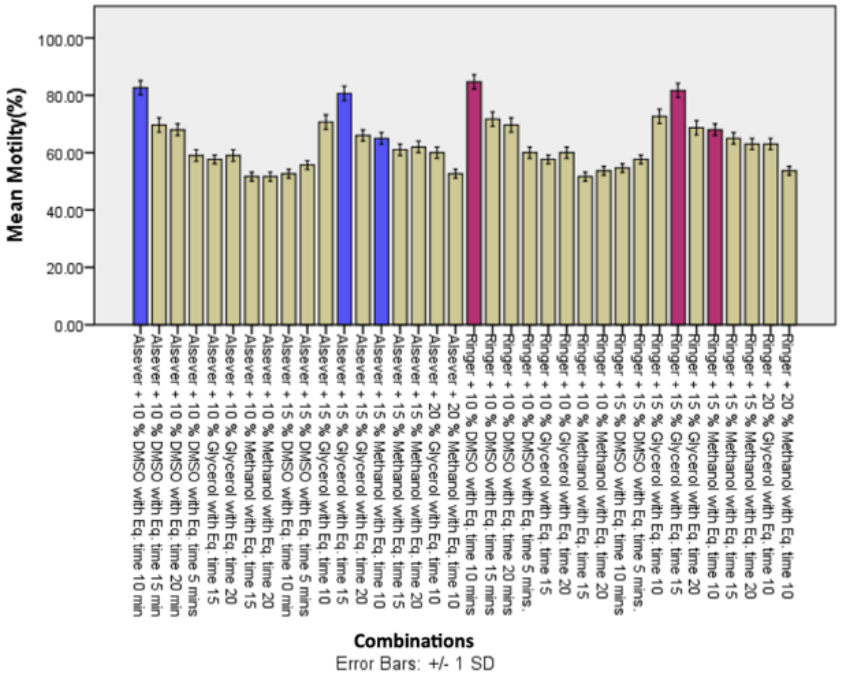

Fig. 2. Mean Motility of Spermatozoa of *Sillago sihama* with extender cryoprotectant combinations & equilibration time at 4°C

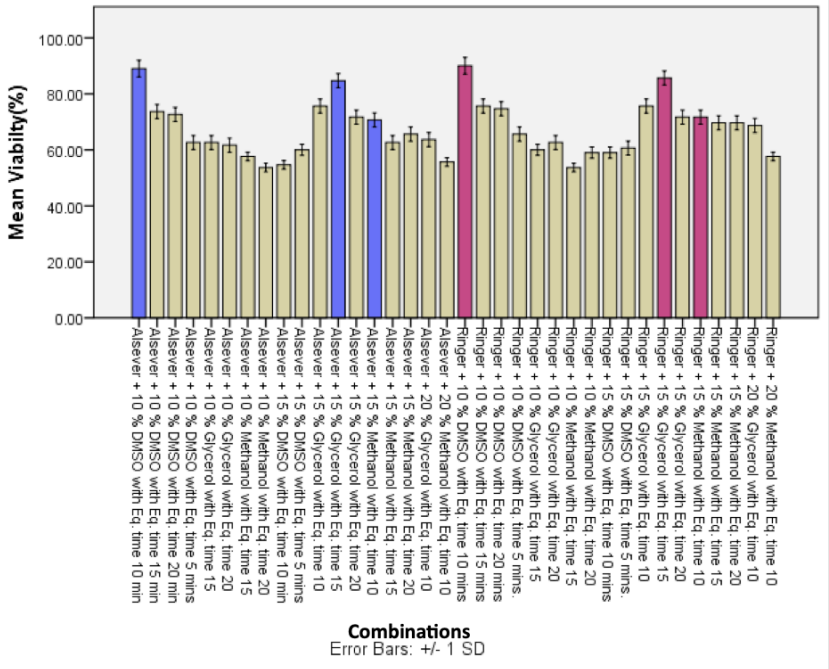

Fig. 3. Mean Viability of Spermatozoa of *Sillago sihama* with extender cryoprotectant combinations & equilibration time at 4°C

(10 %) yielded sperm motility of 71.67 ± 2.08 % and viability of 77.00 ± 3.62 %. Among the selected diluents, Marine Ringer (Burton, 1988) and Methanol (15%) and Alsever's Solution with Methanol (15%) faired the least. Marine Ringer (Burton, 1988) and Methanol (15%) exhibited a motility (49.33 ± 2.52 %) and viability (53.00 ± 2.65 %) and Alsever's Solution with Methanol (15%) exhibited a motility (47.00 ± 1.00 %) and viability (54.00 ± 2.65 %) (Fig. 4).

ANOVA test was also conducted to check the significance of various diluents on motility and viability of spermatozoa after 15 days of storage in liquid nitrogen at -196°C. There was a significant relation ($P<0.05$) between the parameters.

3.3. B 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 %. Alsever's Solution with DMSO (10 %) yielded sperm motility of 51.67 ± 0.58 % and viability of 56.33 ± 0.58 %. Among the selected combinations, Marine Ringer (Burton, 1988) and Methanol (15%) and Alsever's Solution with Methanol (15%) scored the least. Marine Ringer (Burton, 1988) and Methanol (15%) exhibited a motility (33.67 ± 1.15 %) and viability (37.00 ± 2.00 %) and Alsever's Solution with Methanol (15%) exhibited a motility (32.33 ± 2.52 %) and viability (40.00 ± 1.00 %).

ANOVA test showed that there is a significant relation ($P<0.05$) between various diluent used on motility and viability of spermatozoa after 30 days of storage in liquid nitrogen at -196°C (Fig. 5).

3.4. Integrity Assessment

Functional integrity and DNA integrity were also assessed for milt diluted with Marine Ringer (Burton, 1988) and DMSO (10 %), which showed higher motility and viability after 15 and 30 days of storage. Marine Ringer (Burton, 1988) and DMSO (10 %), the spermatozoa of *Sillago sihama* exhibited functional integrity of 70.33 ± 4.51 and 59.33 ± 4.73 after 15 and 30 days of storage. DNA integrity of 89.33 ± 4.51. and 73.67 ± 5.69 was noted after 15 and 30 days of storage (Fig. 6-9).

4. 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 %, a viability of 89.8 ± 4.20 %. Viveiros et al. (2012) reported that the percentage of motile spermatozoa actually corresponds to sperm viability. Higher spermatozoa motility of (97%), a motility duration (164 s) and spermatozoa density ($9.28 \times 10^6 – 10.18 \times 10^6$ nos. ml^{-1}) was observed in *Sillago sihama* in the month of July (Vinod and Basavaraja, 2010).

Toxicity of cryoprotectants on spermatozoa was evaluated by screening different cryoprotectants DMSO, Glycerol and Methanol at varying concentrations (5, 10, 15 and 20%) with Marine Ringer (Burton, 1988) and Alsever's Solution at a dilution ratio of 1:3 at 4°C, using different

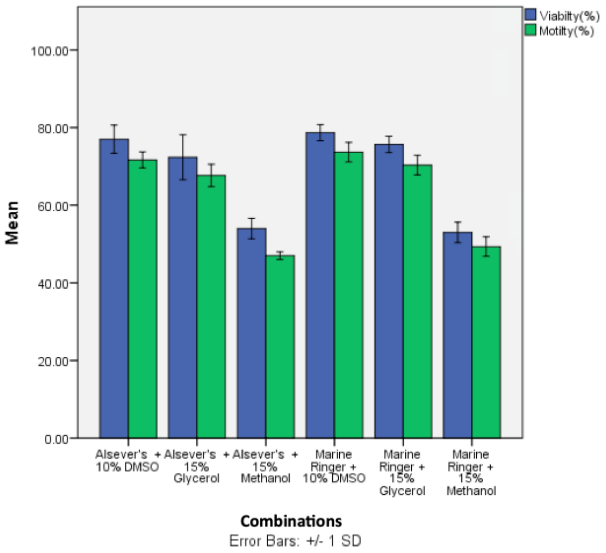

Fig. 4. Mean Motility and Viability of post-thaw cryopreserved sperm after 15 Days of Storage in liquid nitrogen at -196°C

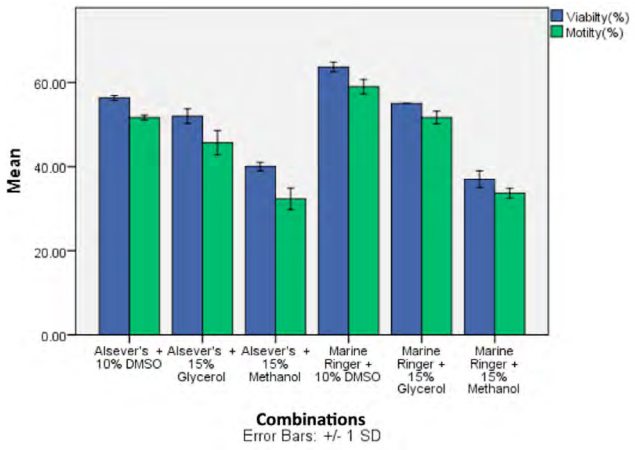

Fig. 5. Mean Motility and Viability of post-thaw cryopreserved sperm after 30 Days of Storage in liquid nitrogen at -196°C

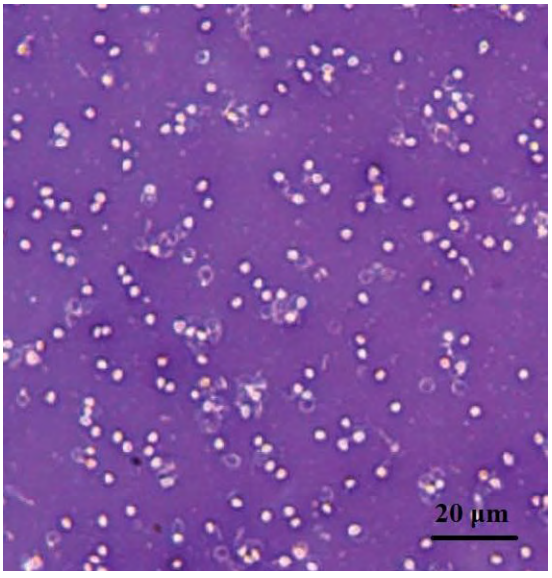

Fig. 6. Control sperm exhibiting maximum tail swelling as an index of Functional Integrity

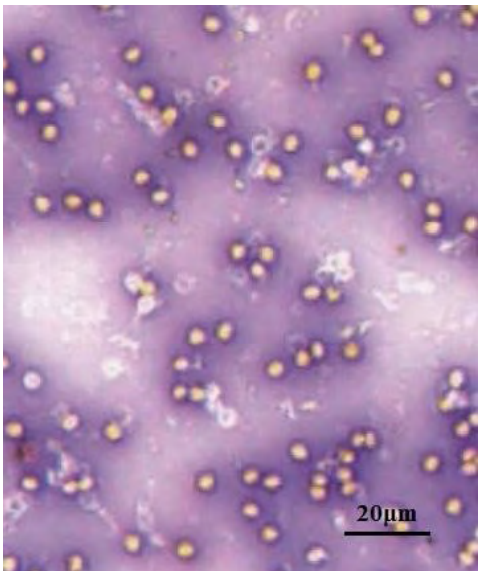

Fig. 7A. Functional Integrity 15 days of storage

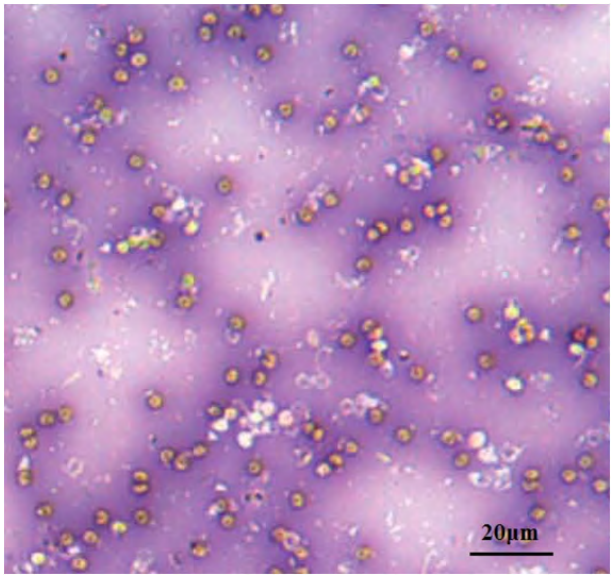

Fig. 7B. Functional Integrity 30 days of storage

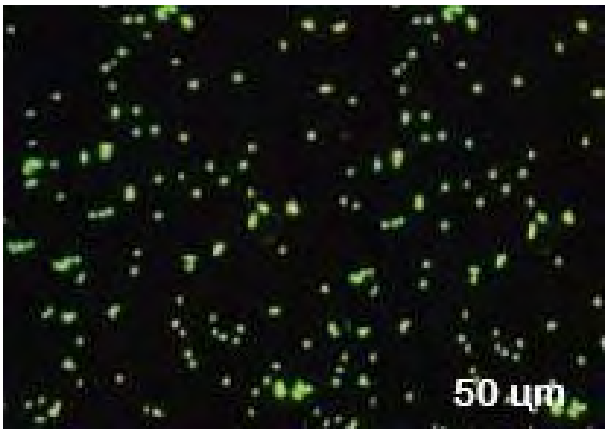

Fig. 8. Control sperm exhibiting DNA Integrity

equilibration time. Marine Ringer (Burton, 1988) with 10 % DMSO at an equilibration time of 10 minutes showed higher motility (84.67 ± 2.52 %) and viability of (90.00 ± 3.00 %), followed by Alsever's Solution with 10 % DMSO at an equilibration time of 10 minutes with a motility and viability of 82.67 ± 2.52 % and 89.00 ± 3.00 % respectively. Studies suggest that the effectiveness of cryoprotectants depends on a relationship between toxicity and the capacity of cryoprotectants to protect the cells (Lahnsteiner et al., 1996 and Muchlisin, 2005). Of the various cryoprotectants tested, DMSO proved to be the best in the presnt study. DMSO generally gave the best results and its success can be explained by the fast penetration into spermatozoa and by its interaction with the phospholipids of the sperm membrane (Baulny et al., 1996). Riesco et al., 2016 also suggested that DMSO is one of the most suitable and efficient cryoprotective agents on account of its rapid ability to penetrate the cells, thereby protecting cells from freezing and thawing injuries. An equilibration time of 10 min seemed to be ideal for *Sillago sihama* sperm. Literature supports that an equlibration time of 10 to 20

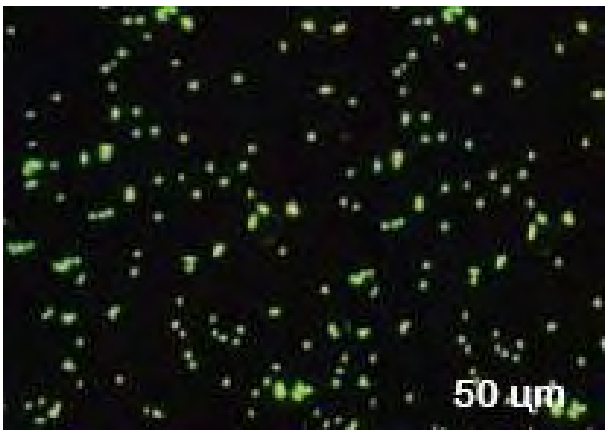

Fig. 9A. DNA Integrity 15 days of storage

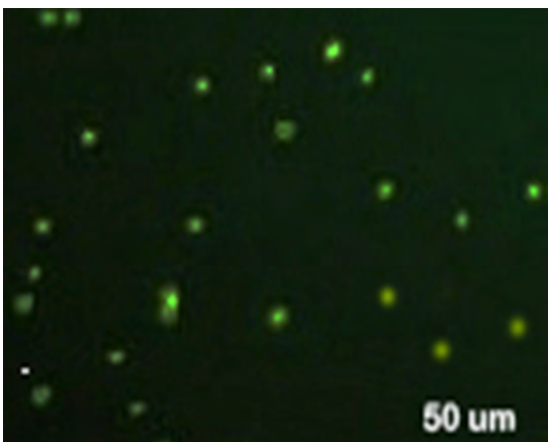

Fig. 9B. DNA Integrity 30 days of storage

Green fluorescence indicates undamaged DNA; red and orange indicates damaged DNA

min is used for fish spermatozoa (Billard and Zhang, 2001). Toxicity assays serve as an important step before designing a cryopreservation protocol. Based on the results of toxiciy assessments, 6 combinations of extender and cryoprotectants were shortlisted for long term cryopreservation for 15 and 30 days. Post-thaw assessment after 15 and 30 days yielded better results for milt diluted with Marine Ringer (Burton, 1988) with DMSO (10%) followed by Alsever's Solution with DMSO (10%). Among the selected combinations, Marine Ringer (Burton, 1988) with Methanol (15%) and Alsever's Solution with Methanol (15%) faired the least. The difference in results with different cryoprotectants may be attributed to the permeability of cryoprotectants and varying toxicity tolerance of sperm to the cryoprotective agents (Cabrita et al., 2003).

The findings of the present study are in agreement with the studies of Tian et al. (2008) and Tsai et al. (2010) that DMSO can be used successfully with spermatozoa of many fish species. With Summer Whiting, *Sillago ciliata* sperm cryopreserved with DMSO and Glycerol retained motility after freezing and thawing and with methanol none of the sperms retained motility and all were dead (Young et al., 1992). Leung (1987) also found DMSO to be a superior cryoprotectant with Barramudi sperm. Chao et al. (1975) reported that DMSO and Glycerol at the 5% or 10% concentrations proved most desirable for Grey Mullet, *Mugil cephalus*. The reason for DMSO being superior may be due to its faster ability to permeate into the cells, bringing a rapid balance in concentrations of intracellular and extracellular fluid (Ciereszko et al., 1993). In the present study, DMSO was found to be the most effective cryoprotectant.

The structural and functional integrity of sperm are the important aspects that correspond to sperm viability (Lechniak et al., 2002). Studies support that the fertility of sperm can be determined by the functional integrity of sperm plasma membrane (Henkel et al., 1993). Eosin and nigrosin staining are used for semen evaluation and detecting abnormal sperm (Herak, 1991). However, HOS test forms a much more promising technique for determining the fertilizability of sperm plasma membrane where biochemically active or dead sperms can be differentiated (Madeja et al., 2003). Functional integrity and DNA integrity were also assessed for milt diluted with Marine Ringer (Burton, 1988) and DMSO (10%) which showed higher motility and viability after 15 and 30 days. Marine Ringer (Burton, 1988) and DMSO (10%), the spermatozoa of *Sillago sihama* exhibited functional integrity of 70.33 ± 4.51 and 59.33 ± 4.73, DNA integrity of 89.33 ± 4.51 and 73.67 ± 5.69. after 15 and 30 days of storage respectively. The present study indicates a significant relation between HOST coiling (%), acridine orange assay, motility (%) and viability (%).

5. Conclusion

The use of cryopreserved fish spermatozoa of *Sillago sihama* would definitely help to overcome the difficulty of having and maintaining mature males for spawning and aquaculture practices. To conclude, the results of the present study represent base-line data for sperm cryopreservation of *Sillago sihama* (Forsskal 1775),

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