India being an agricultural country, its economy mainly depends on the successful production of agricultural products i.e. Crop, Animal husbandry, Poultry and Fisheries. It is one of the richest nations in the world with regard to genetic resources of fishes which are distributed over a network of perennial river systems in the country. Most of the increase in fish production is expected to come from aquaculture which is currently the fastest growing food production sector of the world. As world’s human population continues to expand beyond 6 billion, its reliance on farmed fish production for important source of protein will also increase owing to stagnating production from capture fisheries sector. To meet this growing demand of world population for fish protein, diverse strategies like species diversification, culture of exotic species, incorporation of improved strains in culture system or improved culture practices are needed for producing quality fish on lower cost.

Cyprinus carpio (common carp), an exotic fish species brought from Bangkok (Thailand) in 1956, has become integral part of fish culture system in India. The fish has acclimatized to a wide range of habitats and environmental conditions and therefore enjoys a world-wide distribution. Common carp is an economically important fish species cultured mostly in Asia and Europe. Artificial insemination can be applied to increase the production to meet increasing demand due to exploitation of natural stocks. Cryopreservation is a valuable technique to assist in the genetic improvement of cultured stocks as well as providing a continuous supply of good quality sperm for artificial insemination. Successful storage of fish sperm in liquid nitrogen has been reported for more than 200 species but the protocol varies with species. Extender composition, cryoprotectant concentration and freezing method are known to affect cryopreservation success. Freezing can be performed by programmable temperature changes or simple immersion in liquid nitrogen vapor above the surface of liquid nitrogen. Research on the development of sperm cryopreservation in common carp has not yet addressed on the use of extenders with simplified ingredients of ions and saccharides in combination with various cryoprotectants and freezing methods. Standardization and simplification of cryopreservation procedure for common carp sperm are needed for commercial application. The present study aims to cryopreserve common carp sperm (Cyprinus carpio) with comparative account of effect of extenders and cryoprotectants on motility, viability and fertilization of spermatozoa and comparison of embryonic development of common carp.

**MATERIAL AND METHODS**

Milt was collected from unanesthetised specimens of common carp maintained at the College of Fisheries, Pantnagar. About 20 mature male brooders (2-2.5 kg body weight) of Common carp were collected from Instructional Fish Farm of College of Fisheries. Prior to stripping, the milters were washed with Ringer’s solution and wiped dry around the genital papillae. Diluents for the milt consisted of an extender plus a cryoprotectant in the v/v ratio 9 parts extender: 1 part cryoprotectant in the v/v ratio 9 parts extender: 1 part cryoprotectant.
of 1:4. Carp brood stocks will also be maintained in indoor tanks supplied with a continuous flow of tap water.

**A. Extenders and Cryoprotectants**

**Extenders:** TRIS, RPMI 1640 (SIGMA) culture medium in 0.9% NaCl solution and Phosphate buffer saline.

**Cryoprotectants:** DMSO and Glucose

**B. Experimental design**

Experiment (1) Milt + (TRIS + DMSO)

Experiment (2) Milt + (TRIS + Glucose)

Experiment (3) Milt + (TRIS + DMSO + Glucose)

Experiment (4) Milt + (TRIS)

Experiment (5) Milt + (RPMI 1640 + DMSO)

Experiment (6) Milt + (RPMI 1640 + Glucose)

Experiment (7) Milt + (RPMI 1640 + DMSO + Glucose)

Experiment (8) Milt + (RPMI 1640)

Experiment (9) Milt + (PBS + DMSO)

Experiment (10) Milt + (PBS + Glucose)

Experiment (11) Milt + (PBS + DMSO + Glucose)

Experiment (12) Milt + (PBS)

**C. Cryopreservation:** Freshly collected milt mixed with different diluents were discharged into polyvinyl straws. The straws were immediately sealed from open end, wiped and immediately transferred into biological freezer. The biological freezer holding the straws after allowing varying equilibration period were frozen by exposing them to liquid nitrogen vapour at about the surface of liquid nitrogen for 2-5 minutes, after which the straws were immersed and frozen at -196°C.

**D. Motility and fertility:** The motility of milt was checked by taking a small drop of stored sperm diluted with excess of Hiltfreter’s solution on a glass slide and examined microscopically at an ambient temperature. An arbitrary scoring system modified form was used to assess sperm motility. Sperm motility is expressed on a 6 pt. scale based on the percentages of motile sperm:

<table>
<thead>
<tr>
<th>Score</th>
<th>Condition</th>
<th>Percentage of motile sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>All dead</td>
<td>Zero</td>
</tr>
<tr>
<td>1</td>
<td>Slightly active</td>
<td>1-5%</td>
</tr>
<tr>
<td>2</td>
<td>Oscillating</td>
<td>5-30%</td>
</tr>
<tr>
<td>3</td>
<td>Moving</td>
<td>30-50%</td>
</tr>
<tr>
<td>4</td>
<td>Active</td>
<td>50-70%</td>
</tr>
<tr>
<td>5</td>
<td>Excellent</td>
<td>70-100%</td>
</tr>
</tbody>
</table>

Fertility of preserved milt was tested using evolved eggs stripped from hypothesized females. The ova were poured in large-sized beakers and kept under the running tap. Sample of ova were withdrawn at intervals and examined under a low power microscope.

**RESULTS AND DISCUSSION**

Out of the 12 combinations of three extenders, TRIS+DMSO+Glucose, RPMI1640+DMSO and PBS+DMSO gave satisfactory results. TRIS+ Glucose+ DMSO gave the best result in terms of post thawing motility closely followed by PBS+DMSO and RPMI1640+ DMSO. Maximum motility score was recorded with TRIS+ Glucose+ DMSO. The capacity of the cryopreserved sperms to fertilise eggs was tested on common carp. The motility percentage was highest in TRIS+ Glucose+ DMSO (75%) followed by RPMI1640+DMSO i.e. 67%, PBS + DMSO, 60%. Fertility evaluation conducted by sperm frozen with TRIS+ Glucose+ DMSO on 50 ml of ova gave the highest fertilization rate of 69% and 55% hatching rate followed by RPMI1640+DMSO which gave 55% fertilization rate with 49% of hatching rate and PBS+DMSO gave 48% fertilization rate and 44% hatching rate.

Embryonic development in cryopreserved sperm-fertilized eggs of common carp was observed from 0 hours to 70 hours and results shows that development of blastodisc started in 7 hours and development of embryo began in 11 hours. Differentiation of head region is completed in the developing embryo within 14 hours. Body somites are seen in 22 hours. Differentiation of brain parts are visible in 36 hours, followed by differentiation of eye ball, branchial and vascular bed within 46 hours, while yolk sac absorption within 60 hours. Actively functional digestive system was observed within 70 hours. By comparison of effect of three successful combinations of extenders and cryoprotectants on the basis of pattern of yolk sac absorption, it was found that combination of PBS+DMSO shown normal embryonic development whereas slightly better result was shown in TRIS+DMSO+Glucose and delayed embryonic development in RPMI+DMSO. Also, the result reveals that in case of combination of PBS+DMSO as well as TRIS+DMSO+Glucose, yolk absorption was normal while in RPMI+DMSO combination yolk absorption was slow and late. So, the combination of TRIS+DMSO+Glucose and PBS+DMSO could be used for successful embryonic development.
Photo Plate 1: Embryonic development in cryo-preserved sperm-fertilized eggs of Common carp from 7 hour to 36 hour (A=TRIS+DMSO+ Glucose, B= PBS + DMSO, C= RPMI 1640+ DMSO)
Photo Plate 2: Embryonic development in cryopreserved sperm-fertilized eggs of Common carp after 46 hour to 70 hour (A=TRIS+DMSO+ Glucose, B= PBS + DMSO, C= RPMI 1640+ DMSO)
Long-term storage involves the use of diluting media and protective agents. The media in which fertilization naturally occurs didn’t enhance the spermatozoan survival. A formulation with three items viz. Sodium chloride for tonicity, Sodium bicarbonate for buffer and vegetable lecithin to protect membrane. Other substance tried were TRIS, PBS, Magnesium chloride, Egg yolk, Urea, Mannitol, Fructose and Dextrose. Among the protective agents the use of Ethyleneglycol and Dimethyl sulfoxide have been reported. The best result have however, been achieved with DMSO. The present study was mainly aimed towards comparison of extenders and cryoprotectants for the survival of carp sperms under cryogenic conditions. Amongst all the combinations, TRIS+DMSO+Glucose, RPMI 1640+DMSO and PBS+DMSO gave satisfactory results but these were not consistent and cases of coagulation of thawed milt were quite common. As stated earlier, the incidence of coagulation of thawed milt was quite high with extender TRIS. Several reasons have been attributed by various workers for these clump formation after thawing the stored milt viz. improper concentration of the milt prior to their immersion in liquid nitrogen. Along with the cryogenic preservation, motility 90% was recorded till one hour after storage thereafter it gradually declined. Storage of undiluted Rainbow trout milt for at least 23 days prior to loss of fertility. The motility score of 30-90% of diluted samples after storage period of 20 hours in the present study is quite significant as it shows that carp milt can be stored without adding any extender. Though there are several ways to detect live sperm viz. motility, differential staining the fertility evaluation test is considered more accurate. The few trials conducted indicated that swirling sperm samples always give high fertilization rate while the fertilization done with negligible or even 20% motile sperms never fertilise the eggs. The loss of activity in the sperm may be either due to loss of locomotion in which case the sperms are unable to find or penetrate the micropyle or to a general deterioration, as the capacity of the fertilization is lost.

The present study concludes that observations are indicative of successful cryopreservation of common carp sperm by using either one of TRIS, RPMI1640 and PBS as extender with the addition of DMSO and Glucose together as cryoprotectants. The use of TRIS diluent with DMSO and Glucose is rated as the best combination for freezing common carp sperm. The formation of sperm agglutination in common carp sperm is likely affected by extender composition, type of cryoprotectant and cooling conditions. Thus, the use of TRIS, RPMI1640 and PBS with DMSO and Glucose for freezing common carp sperm in the liquid nitrogen vapour may have paramount importance being a viable protocol for captive breeding program in aquaculture. In case of embryonic development, TRIS+DMSO+Glucose and PBS+DMSO gave satisfactory result.

REFERENCES