Chapter

Cryopreservation and Its Application in Aquaculture

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Abstract

Aquaculture is the major aquatic animal production segment. Problems like inbreeding depression, genetic drift, introgressive hybridization, etc. have been influencing the production of quality seeds negatively. Cryopreservation serves as a way-out for these problems and a possible answer to produce quality seeds and genetically improved varieties. It has been considered as a major strategy for conservation of fish genetic resources. Cryopreservation of fish gametes has been in vogue since 1953 and the technology is well studied and validated for many species. So far the milt of 200 fish species has been cryopreserved successfully. In this chapter, the importance of aquaculture in overcoming malnutrition, genetic issues affecting quality seed production, cryopreservation protocol employed for various fish species, problems faced in cryopreserving fish eggs and embryos and future of cryopreservation in aquaculture have been discussed.

Keywords: Cryopreservation, aquaculture, fish, spermatozoa, egg, inbreeding

1. Introduction

In the World Summit on Food Security held at Rome during 16–18 November 2009, it was committed to eradicate hunger from earth by increasing investment in agriculture involving public and private enterprises. Food and Agriculture Organization (FAO) defined food insecurity as a situation that exists when people lack secure access to sufficient amounts of safe and nutritious food for normal growth and development and an active and healthy life.

The projections of FAO for the next 15 years indicate that, if agricultural innovation continues at a reasonable rate, food production can increase by 2 percent per year in the developing countries. Another report by World Bank mentioned that the world needs to produce at least 50% more food to feed 9 billion people by 2050. If the natural resources offer good potential for agricultural development, supporting agriculture research can bring big benefits in reducing food insecurity and malnutrition [1]. In solving the above issues, along with grains and vegetables, fish can also play a major role.

Fish and other aquatic products provide at least 20% of protein intake for one third of the world's population and the dependence on fish is high in developing countries [1]. Small-scale fisheries are considered to be more important for food security

because they supply more than half of the protein and minerals for over 400 million people in the food deficit countries of Africa and South Asia [2].

Fish is not an energy food, but it is an essential food for the human being. It is an extremely important source of protein, minerals and oils in many under developed countries. Fish protein constitutes around 30% of the Micronesian diet and 15% of the Polynesian diet [2]. Fish is more nutritious than other staple foods, providing quality animal protein, essential fatty acids and micronutrients. Interventions related to fish intake and aquaculture production include, utilizing fish as complementary food to improve nutritional status of children, encouraging children and women to eat nutrient-dense fish through nutrition education at community level [3] and increasing production of more demanded fish species through effective dissemination of the technology [4].

Fish being an important food for mankind, its production through all possible means has been explored. Besides exploitation of natural resources like sea and natural water impoundments, culture production through structured methods deserve due attention now. This shift in the population in the natural waters has impacted the availability of fish through capture and driving the people to develop ways to produce the fish through aquaculture. Nevertheless, the impacts of climate change on aquaculture also could not be pushed off.

Studies in Asia suggest that low-income households consume lesser quantities of fish than rich households [5], but they still depend on fish as a major source of animal protein [6]. This invariably suggests that fish supply should be sufficient to wade off the malnutrition from the low-income group of people in order to keep the life free from nutritional disorders.

It is at this point anthropologists in the world insist that apart from concentrating on improving agriculture production, agencies like FAO and World Bank must establish projects in aquaculture of species that are positively impacted by climate change and at the same time contains high nutrients to eradicate malnutrition. Some of the fishes rich in nutrition include carps, catfish, murrels, tilapia and prawn [7].

Globally, fish provides 20% of average per capita intake of animal proteins to more than 3.3 billion people. In some countries like Bangladesh, Cambodia, the Gambia, Ghana and Indonesia, fish contribute to 50% or more of animal protein. The global fish consumption per capita food grew from 9.0 kg (live weight equivalent) in 1961 to 20.5 kg in 2018 which is about 1.5% increase per year [8].

The total world fisheries and aquaculture production has reached 179 million tonnes in 2018 which was recorded as highest of all times and estimated at USD 401 billion [8]. The aquaculture sector was the main driver that led to the increase in production of aquatic animals, and the total aquaculture production was estimated to be 82.1 million tonnes valued at USD 250 billion with average growth of 5.3% per year. The contribution of world aquaculture to global fish production has increased from 25.7% in 2000 to 46% in 2018.

2. Genetic issues affecting seed quality

Aquaculture in many countries depends on the adequate supply of quality seeds. However, it is not always possible since many hatcheries have reported stock deterioration due to poor brood stock management, inbreeding depression, genetic drift, introgressive hybridization, unconscious selection, lack of effective population size (N_e) and genetic erosion of domesticated stock.

2.1 Prevention of detrimental effect of inbreeding and genetic drift via cryopreservation

Inbreeding and genetic drift cause undesired changes in the genome and result in lower viability and growth and increase developmental instability in fishes [9]. Unplanned and uncontrolled breeding often lead to inbreeding depression which lead to decreased growth rate, low fecundity and poor survival which are usually accompanied by loss of alleles via genetic drift [10]. Uncontrolled inbreeding and genetic drift occur together in closed hatchery populations and these factors are determined by the population's N_e. Hence maintaining the desired N_e will prevent adverse effects on productivity and profits [10].

Due to high fecundity in fishes, inbreeding is more prevalent in aquaculture than other domesticated animals. This applies especially to highly fecund species like Indian Major Carps (IMC) (catla, *Catla catla,* rohu, *Labeo rohita,* mrigal, *Cirrhinus mrigala*) and Chinese carps (silver carp, *Hypophthalmicthys molitrix*, grass carp, *Ctenopharyngodon idella*, common carp, *Cyprinus carpio*) where few broodstock are necessary to meet demands for fry and broodstock replacement. The detrimental effects of inbreeding are well documented and can result in 30% or more decrease in growth, survival and reproduction [11].

The problem of inbreeding and genetic drift can be reduced by spawning more fish than needed. Since the fecundity is high in some species, the required number of fingerlings can be produced by breeding one or two females and males. But the ability to spawn relatively few fish must be moderated if inbreeding and genetic drift are to be controlled. Another way to increase N_e and reduce the rate of inbreeding and genetic drift is to spawn a more equal sex ratio. Most farmers and hatchery managers use skewed sex ratios when they spawn their fish. This is done because one male can usually be used to fertilize eggs from several females. This enables farmers to use and maintain fewer males, which lowers production costs. When a skewed sex ratio is used, the rarer sex has a disproportionate influence on the size of N_e. Cryopreservation can help in maintaining N_e by breeding more number of fish which leads to maintenance of fewer males in the hatchery [10].

2.2 Introgressive hybridization with cryopreservation

It is the gradual infiltration of the germplasm of one species into that of another as a consequence of hybridization and repeated backcrossing [12]. Sarder et al. [13] reported that pure mrigal are severely being threatened by introgressive hybridization in Bangladesh. Moreover, unplanned hybridization, inbreeding depression and genetic drifts have been the causes of deteriorating quality of this species. They opined that cryopreservation is the simplest and most inexpensive method to preserve genomes that can be used to maintain future conservation options. Introgression of autochthonous populations with introduced ones is a common phenomenon in salmonids and it can result in outbreeding depression and replacement of possibly locally adapted populations by allochthonous ones [14].

Horvath et al. [15] applied cryopreservation as a conservation effort of two salmonid species such as the marble trout (*Salmo marmoratus*) and the Adriatic lineage of the grayling (*Thymallus thymallus*) autochthonous to the drainage of the Soča river in Slovenia. Populations of these species were greatly affected by hybridization and introgression with allochthonous species such as the brown trout (*Salmo trutta m. fario*) and the Danubian lineage of the grayling that were introduced to the

Soča drainage during the 20th century. Cryopreservation of sperm from the Adriatic grayling and the marble trout has constituted an integral part of the conservation activities. In case of the grayling, no pure population was available and hence the proportion of Adriatic genotype in the broodstock was increased. Genetic analyses of the populations were also conducted. Sperm and fin clips were collected from wild males on the spawning grounds. Sperm was cryopreserved and stored until the genetic analysis was completed on each sample. Cryopreserved sperm of individuals containing higher than a pre-defined proportion of Adriatic genotype was thawed and used for fertilization of eggs from Adriatic females. The resulting progeny was developed as broodstock and 70–80% of the local grayling broodstock originated from cryopreserved sperm. In case of the marble trout, cryopreservation was used to create "sanctuary" streams. Sperm is collected from wild males of a given pure population prior to the spawning season (early November) and cryopreserved. Sperm is stored in liquid nitrogen (LN_2) until the spawning season (December–January) and then eggs of females from the identical population are fertilized with the cryopreserved sperm. Eyed eggs are then stocked into artificially created nests in the prepared "sanctuary" stream. Thus, a high number of males of the given pure population participate in the creation of the new population.

From a management perspective, the desirability of introgressive hybridization in response to environmental change depends on the circumstances. It is desirable when the resulting adaptation has the potential to rescue a native species from extinction, such as adaptation to the sudden climate shifts that might become more frequent or extreme with climate change. In such cases, management actions to protect hybridization, such as the protection of hybrid zones, might enhance the potential for species to respond to environmental change [16].

3. Aquaculture and cryopreservation

Fish breeding depends on many factors and failure or partial success in the breeding is a reality for many successful hatchery operators. In order to get the required quantity of seeds, induced breeding is considered as a viable tool that makes the fishes maturing and spawning despite to low or poor rainfall and worst climatic conditions. Nevertheless, the health of brooders is severely affected by repeated breeding attempts within its confined life time. Exchange of brooders is not a simple task due to difficulties and physiological factors associated with the transportation of the brooders. Therefore shipping of gametes is considered as a possible alternative that may have its own advantages as witnessed in the animal husbandry.

It is necessary to introduce biotechnological tools in fish breeding programme to ensure continuous seed production. Cryopreservation may be a possible answer to produce quality seeds and genetically improved varieties. FAO has endorsed cryopreservation as a major strategy for conservation of fish resources [17]. Cryopreservation increases the longevity of gametes for several years without any drastic change in the fertilizing capacity of the gametes by lowering the temperature usually to −196°C [18] which arrests all biological activities, including biochemical reactions that lead to cell death and DNA degradation [19].

In fishes, Blaxter [20] is believed to be the first successful scientist who did the cryopreservation of herring spermatozoa and proceeded up to artificial fertilization with the cryopreserved spermatozoa. It has been reported that so far milt from over 200 species of freshwater and marine fish have been cryopreserved [21, 22].

4. Principle of cryopreservation

The basic principle of cryopreservation is exposure of living cells to sub-zero temperature as low as −200° C through a perfect process thereby arresting its activities without damaging the life of it. A series of complex and dynamic processes of heat and water transport between cells and their surrounding medium is involved during the freeze–thaw process of biological material. The effect of the process depends on the speed at which the cells are frozen or thawed. When cells are frozen in an aqueous solution, both cells and the solution get super cooled leading to freezing that will be followed by heterogeneous nucleation, usually in the extracellular solution. The same condition can be seen in the cell solutions also. If such condition occurs intracellularly, the resultant nuclei will be isolated by plasma membranes from the unfrozen cell components and leads to separation of ice crystals inside the cell. As water gets frozen, the extracellular solution becomes progressively more concentrated leading to slow dewatering conditions in the cells. This results only when the cooling is slow and there is sufficient time for the cells to lose enough water so as to remain in osmotic equilibrium with the concentrating extracellular solution leading to water loss inside the cells. If that occurs, that will lead to cell death otherwise called as freeze killing or chill killing. While this may take time in large and multi cellular organisms, in small micro-organisms and single cells much of water can be withdrawn during freezing leading to desiccation and the death of the cell instantly [23].

In contrary to the above situation, if the rate of cooling is faster and rapid, there will be less time for the intracellular water to diffuse out of the cells. A balancing situation will emerge under such fast or rapid cooling. This leads to survival of the cells by minimizing the time or exposure duration to concentrated solution. The cooling rate also ensures there is no formation of intracellular ice. This process is called vitrification and it is the process that is happening inside the cell in the cryopreservation process [23].

During thawing, the same cellular physiological processes occur in reverse order. The thawing rate should also be rapid and fast enough that of the corresponding cooling rate. Nevertheless, recrystallization invariably occurs during thawing, forming lethal intracellular ice. A high warming rate is usually employed to minimize the degree of recrystallization when thawing is rapid to provide insufficient time or least possibility for the dehydrated cells to absorb the amount of water lost during freezing [23].

5. Cryopreservation of fish spermatozoa

5.1 Milt collection

Cryopreservation success depends on the milt quality and hence, quality of milt must be evaluated based on the condition of spermatozoa prior to cryopreservation. Milt should be always collected from oozing ripe brooders by stripping method in ice cold, sterilized cryovials [19]. Milt must be collected in clean, dry and sterile vials and immediately stored on ice [19]. Collected milt should be in quiescent form and should be free from contaminants, such as water, mucus, blood, and gut exudates. Prior to stripping, the urinary bladder can be emptied by gentle squeezing in order to avoid milt contamination with urine.

Employing stripping method for collecting fish milt might result in contamination with urine which may seriously influence milt characteristics and quality [24]. The contaminated milt can deteriorate the spermatozoa quality and have detrimental effects on post thaw viability as the contaminants such as urine, blood, mucus, etc. can change the seminal fluid composition and induce sperm motility [25]. Urine contamination can lead to lower percentage of fertilized eggs [26].

Using a catheter for milt collection can avoid urine and fecal contamination [27–29]. Researchers have suggested that anesthetizing the donors prior to milt collection was advantageous [30, 31]. Anesthetizing agents like Tricaine methane sulphonate (MS-222) can be used before milt collection [32]. Fish can be anesthetized by immersing in 2-phenoxyethanol for 2 min at a dose of 0.5 ml/l of water [33].

For instance, *O. mykiss* was anesthetized with MS 222 in a 1:10,000 dilution water bath during milt collection and the milt was collected by gently massaging the abdomens of the fish [34]. *C. carpio* brooders were anesthetized with a 1:1000 aqueous solution of 2-phenoxyethanol before handling [35]. *C. carpio* males were anesthetized with 2-phenoxyethanol at a dose of 0.5 ml before milt collection [36].

5.2 Spermatological properties

Sperm quality evaluation is very important as it provides necessary information for optimal handling and storage protocols for sperm used in artificial fertilization [25, 37]. The fish milt composition and its physical characteristics vary with species and are important from the aspect of milt quality [38]. The quality of the milt is species specific [39] and can be affected by the feeding regime, feed quality, rearing temperature and spawning season of males [40, 41]. Spermatozoa motility, milt volume and the spermatozoa concentration are considered to be good indicators for milt quality [42, 43]. Sperm quality can be evaluated based on the sperm volume, spermatozoa density, motility of spermatozoa [25]. The appearance, color and nature of milt are also used to assess the quality of milt. The milt volume of fishes is found to vary with species [44–46].

5.2.1 Sperm motility and motility duration

Motility is one of the most important parameters which is most frequently used to assess milt quality after cryopreservation and generally presents a positive correlation with fertilizing capacity [47]. Sperm motility is considered as the best biomarker of milt quality [48]. Motility depends on various aspects of the cell, such as the physiological state of the mitochondria, ATP production, plasma membrane channel integrity and flagellum structure [49]. Relationship between percentage motility and fertilization capacity of spermatozoa was reported in many fishes [50–55]. The motility, velocity and fertilizing ability of sperm was found to vary according to seasonal variations in osmolality of seminal plasma [56–58]. Sperm motility was also found to vary in vigor and duration among individual male depending on ripeness [45].

The spermatozoa are in immobile phase before ejaculation and it was reported that the osmolarity and ion content of the aquatic medium are central factors in activating motility [59, 60]. It was observed that in some of the fish species, the changes in the osmotic pressure (0–300 mosmol/l) could initiate spermatozoa motility [61]. In carp testes and seminal plasma, inhibition of sperm motility was observed due to high osmolality (approximately 300 mosmol/kg) surrounding spermatozoa [50]. Various researchers observed that the spermatozoa usually remain motile for

less than 2 min and sometimes they are only highly active for less than 30 s in most of the freshwater fishes [62–64].

5.2.2 Sperm pH

Milt pH can affect spermatozoa motility and maturation [25]. Hence determination of variation in sperm pH provides information on fertilization capacity of spermatozoa. The milt of most of the freshwater fish species exhibit slightly alkaline pH [65]. When intracellular pH is below 7.5, sperm cells remain immotile with low respiration rate, but in response to an internal alkalinisation, they become motile, concomitantly with an increase in oxygen consumption [66, 67]. The initiation and duration of sperm motility is influenced by the extracellular and intracellular pH [68]. The external pH affects intracellular proton concentration which modifies the membrane potential and motility behavior [69].

5.2.3 Sperm density

Traditionally the density of sperm has been used for the assessment of milt quality. It is an important parameter which has an impact on fertilization success and is a characteristic feature of fish species [70]. Spermatozoa density is usually reflected by sperm volume [71]. Various methods like using Sysmex Microcell counter CC-120 [72], spectrophotometric method [73], haemocytometric method [74] were employed to estimate sperm density in fishes.

5.3 Extender

For successful cryopreservation, it is essential to prevent activation of spermatozoa during preservation. Undiluted milt is unsuitable for storage at cryogenic temperatures, so it should be diluted with an appropriate medium [51]. Because motility of fish spermatozoa is mostly a one-time event, this medium should not induce motility and at the same time must not interfere with the ability of the spermatozoa to be activated subsequently during utilization. Media that satisfies these conditions is called "Extender" [75].

Extender is a salt solution which helps to maintain the viability of cell during cryopreservation, which supplies sources of energy to sperm cells, protect the cells from temperature related damage, and maintain a suitable environment for the sperm to survive during the period of cryopreservation [76, 77]. Based on the inorganic composition of seminal plasma, extender is prepared as a buffered physiological saline solution [78] and hence extender composition differs between species. Extenders maintains the inactivity of spermatozoa when milt is diluted before freezing due to stabilization of physicochemical properties [79]. Sperm typically need to be maintained in an extender with proper osmolality (usually nearly isotonic to the plasma osmolality) to inhibit undesired sperm activation during refrigerated storage or cryopreservation [80].

A large number of extenders such as Ringer's solution, Cortland's solution, Alsever's solution, etc. have been tried for the cryopreservation of spermatozoa of fish which were proven successful for milt cryopreservation in mammals [29]. Several simple extenders which are isotonic in nature, with inorganic salts like NaCl, KCl, $CaCl_2$, $NaHCO_3$, $NaHPO_4$, $MgSO_4$, $MgCl_2$ and others with organic compounds such as fructose, mannitol, lecithin, glycine have been used with varying levels of success [29]. Extenders have been developed using saline and sugar-based diluents [81].

Tris-egg yolk gave higher post-thaw motility percentage (50%) during cryopreservation of milt of *C. carpio* and *L. rohita* [82]. Use of glucose-based extender containing 10% dimethyl sulfoxide (DMSO) could be successfully used for *Oncorhynchus mykiss* milt cryopreservation and fertilization rate similar to that of fresh spermatozoa can be achieved [45]. Sperm diluted with 0.3427 g NaCl, 3.4314 g sucrose, 100 ml DW, 21 μl NaOH solution, 0.5 ml antibiotic (10,000 unit/ml penicillin and 10,000 μg/ml streptomycin) and DMSO gave the best post-thaw motility (94.5 ± 3.3%) in *C. carpio* [83]. The feasibility of three extenders namely, Freshwater Fish Saline, Modified Fish Ringer and Physiological Saline was compared in cryopreserving *C. carpio* milt and the motility duration obtained was 57.28 ± 9.21 s, 64.78 ± 8.84 s and 67.39 ± 4.79 s for Physiological saline, Freshwater Fish saline and Modified Fish Ringer respectively [84].

5.4 Cryoprotectant

Cryoprotectants are low molecular weight compounds that penetrate cells and lower the freezing points of solutions. Cryoprotectants in combination with an effective dilution ratio can also improve the cryo-resistance of spermatozoa [85]. Cryoprotectants need time to penetrate to the cells (equilibration), however, prolonged exposure before cryopreservation can be toxic for sperm [85]. At higher concentrations, cryoprotectants can suppress most of cryoinjuries but at the same time, it can become toxic to the cells [86]. Therefore, suitable cryoprotectant concentration is needed for the development of cryopreservation protocol. The protective effect of cryoprotectants varies in different fish species [87].

Cryoprotectants are very essential for the survival of spermatozoa during cryopreservation. There are two different types of cryoprotectants; permeating and non–permeating [88]. Permeating cryoprotectants such as DMSO, glycerol, methanol, propanediol etc., are believed to lower the freezing point of the solution, which minimize osmotic shock by replacing the water inside the cell, and reduce formation of destructive intracellular ice [89]. Non-permeating cryoprotectants include protein like milk, egg yolk, bovine serum albumin (BSA); sugars such as glucose, sucrose; synthetic polymers like polyethylene glycol and polyvinylpyrrolidone and are believed to stabilize the membrane during cryopreservation [90]. Use of insufficient cryoprotectant before cooling reduces effectiveness, whereas excessive cryoprotectant causes osmotic swelling and rupture during thawing and dilution [91]. Cryoprotectants were found to prevent the formation of ice crystals during freezing [48].

Due to ice crystal formations at low temperatures very few spermatozoa survive without cryoprotectant and same levels of those cryoprotectants can be lethal to unfrozen cell [92]. Cryoprotectants were most effective when they could rapidly penetrate the cell during freezing, which resulted in delay in intracellular freezing and led to minimization of the solution effect [93]. Common cryoprotectants used for fish sperm include DMSO, methanol and propylene glycol (PG) [94].

Regarding these cryoprotectants, PG used for sperm cryopreservation in yellowtail flounder (*Pleuronectes ferrugineus)* resulted to be an effective cryoprotectant [95] but showed moderately good post-thaw motility in *Clarias gariepinus* [96]. Methanol at 10% was found suitable for cryopreservation of bitterling milt [97], bagrid catfish [98] and *C. gariepinus* [99] and 5% methanol was reported to be suitable for tilapia (*Oreochromis niloticus*) milt cryopreservation [94]. DMSO was established to be very successful for cryopreservation of sperm in various freshwater species [100, 101] and has been considered as a universal cryoprotectant [102, 103].

The milt of *C. mrigala* when cryopreserved with glucose as co-cryoprotectant at 0.5% concentration egg yolk at 10% concentration gave the highest post-thaw motility duration [104, 105]. BSA at 2% gave the highest post-thaw motility duration in *C. carpio* [106].

5.5 Dilution ratio

The process of milt dilution is carried out as a means to increase the number of eggs that can be fertilized with a small volume of milt [107]. In fish spermatozoa cryopreservation, dilution of the sperm fluid is one of the most important steps which has been reported to improve fertilization rate as compared with results obtained with undiluted milt [108]. Milt dilution ratio is very important for fish sperm to survive after cryopreservation [109]. The dilution process is very important to increase the volume of milt, so that it can be used for multiple inseminations. Milt is generally diluted 3–20 folds for Salmonid, carp and tilapia [24].

In Cyprinids, full sperm motility is activated at osmolalities <50 mosmol/kg [50]. Using cryopreserved milt, full activation of sperm motility was obtained at ratios of milt to fertilization media of 1:10 for all types of media since at this ratio, the osmolality of the extender–water mixture was high enough to stabilize sperm viability [110]. Too low dilution ratio do not activate full sperm motility and too high ratios results in insufficient low sperm concentrations in the fertilization solution [110]. However, reports also suggest 1:25 [111, 112] and 1:20 [113] as the optimal ratio of milt to fertilization medium.

In this regard, when *C. carpio* milt was diluted with Kurokura medium at 1:5 ratio, it gave best results [114]. Dilution ratio of milt to extender of 1:7 resulted in highest hatching rates while at lower (1,3) and higher dilutions (1,10) fertility was significantly decreased in bleak (*Chalcalburnus chalcalburnus)* [110]. When dilutions of 1:25, 1:50 and 1:100 were evaluated on European perch (*Perca fluviatilis)*, best result was obtained at 1:50 dilution [115]. The highest mean post-thaw motility duration, motility score, percentage of fertilized eggs, and hatching rate was obtained with 1:40 dilution ratio in *C. carpio* [116].

5.6 Equilibration period

Equilibration period is the optimum time that must be allowed to facilitate the penetration of permeating cryoprotectants into the cells while minimizing the toxicity for effective protection during freezing [117].During cryopreservation of milt, an equilibration time of 45–60 min for IMC [118], 10 min for *C. carpio* [119], 60 min for *Tor putitora* [120], 5 min for bleak (*C. chalcoides*) [100], 10 min for *L. rohita* [121], 10 min for *O. mykiss* [122] has been employed with successful results.

Salmo gairdneri milt stored for 20 min after dilution gave significantly higher percentage of fertilization than that stored for 65 min or longer and therefore, cryopreservation of milt should be done as soon as possible after the collection [123]. Poor post-thaw motility was recorded in *C. carpio* at 20 min of equilibration time than that frozen immediately [124]. When the diluted milt was equilibrated for 15 min, there was no adverse effect on the post-thaw fertility of Salmonid milt [48].

5.7 Freezing

Too high freezing rate result in the formation of small ice crystals within the cell due to limited time for the free water to separate from the cytoplasm which

puncturescell membraneand the membranes of the cell organelles. Too low freezing rate exposes the cell to the concentrated cytoplasm for a long time resulting in pickling effect and the biomolecules in the cell get denatured due to the high salt concentration and subsequent changes in the pH [18].

The optimum freezing rate is a moderate rate between the two extremes of the freezing rate [125] which depends on cell type and size, cryoprotectant type and concentration, equilibration time, final temperature prior to plunging in LN_2, fish species and associated interactions [99, 126]. Optimal cooling rate should be rapid enough to minimize the duration of exposure to prevent the occurrence of concentrated solute and slow enough to allow water osmosis to prevent intracellular ice crystal formation [127].

The freezing rate is a critical factor and it was reported that instant immersion in LN_2 may significantly decrease the post-thaw motility duration of fish spermatozoa [128]. Freezing can be performed by programmable temperature changes or simple immersion in LN_2 vapor above the surface of LN_2 [83]. Freezing can also be done using methanol-dry ice bath [129] or by freezing the extended milt with cryoprotectant over crushed dry ice [130]. The pelletization technique in which specific volumes of diluted milt is placed over dry ice (solid CO_2) also served to freeze the milt and it was used by many workers [32, 131, 132].

In a protocol, straws were frozen for 4 min on a stainless steel tray (−80°C) suspended over LN_2 and was immersed into LN_2 [133]. When *C. carpio* milt was frozen 3 cm above the surface of LN_2 for 3 min before plunging in LN_2 it resulted in high post-thaw motility as well as fertilization and hatching rate [134]. During cryopreservation of *C. carpio* milt, the 0.5 ml straws were placed horizontally onto a 3 cm high styroframe raft (−130°C) for 20 min, which was floating on the surface of LN_2, before immersing the straws into LN_2 and it did not negatively affect the fertility of frozen–thawed sperm [35].

Programmable freezers was also used by many researchers for freezing the diluted milt samples of several fish species [135–137]. Programmable freezing allows the pre-setting of different freezing programs, the monitoring of precise temperature during the cooling sections and the continuous biological examination of cells during the freezing stages [138]. Different programmes and different final temperatures can be attained in programmable freezer [119, 139–141]. The use of programmable freezer allows the evaluation of spermatozoa motility at different rates of cooling during freezing [141]. Incorporation of fast freezing rates using the controlled-rate programmable freezer was successfully used in earlier studies for cryopreservation of carp sperm [111, 113, 142].

For cryopreservation of milt of *C. carpio,* the most efficient freezing rate was 5°C/min from 2°C to −7°C and 25° C/ min from −7° C to −70°C [135]. A slower cooling rate at 4°C/min from 0^0 C to −4°C and 11°C/min from −4°C to −80°C can also be used for cryopreservation of *C. carpio* milt successfully [113]. A cooling program of 4°C to −9°C at a rate of 4°C/min and then from −9° C to −80° C at a rate of 11°C/min, which was held for 6 min at −80°C, and transferred into LN_2 was followed for *C. carpio* and high motility (69 ± 14%) and moderate fertilization rate (56 ± 10%) was reported [111]. *C. gariepinus* spermatozoa can be frozen at the rate of −5°C/min initially from +5°C to −35°C and then from −35°C to −50°C or − 70°C [99].

Three different cooling methods were employed during the cryopreservation of *C. carpio* [142]. Two of them used 3 steps, initially from 2°C to −7°C then −7°C to −30°C and finally −30°C to −80°C with two different cooling rates (3 and 6°C/min) after which the sample was transferred to LN_2. In the third method, a one-step method

(2°C to –50°C) with faster average cooling rate (10°C/min) was applied and was reported that faster cooling rates (6 and 10°C/min) were more efficient for cryopreservation and the highest fertilization recorded with 10°C/ min was 99%.

5.8 Thawing

The rate of thawing is an important step which is said to be a decisive factor for the success of cryopreservation procedure. It is the reverse of freezing but rapid thawing after the cooling procedure is preferred however, too high and too low rates of thawing are detrimental for the cryopreserved spermatozoa [18]. Thawing rates should be high enough to avoid recrystallization as its rate is very critical for preservation of spermatozoa viability [143]. It appeared that the ideal thawing procedure almost avoided or reduced either recrystallization and ice crystal formation during thawing. The temperature change should allow movement of water and cryoprotectants while preventing intracellular ice recrystallization [144].

In Cyprinid fishes, the highest mean fertilization percentage of 57% was obtained in *C. idella* when thawed at 20°C quickly in a water bath [145]. The cryopreserved milt of freshwater carps (*L. rohita*, *C. carpio*, *Puntius gonionotus, C. idella*, *Aristichthys nobilis* and *Pangasius sutchi*) was thawed by swirling the frozen ampoules in tap water at 29°C [146]. Similarly, the frozen milt of IMC and *H. molitrix* was thawed by swirling the straws in tap water at 30°C [147]. High post-thaw motility percentage of 92–98% and high hatching percentage of 25.7% was obtained after thawing the cryopreserved milt of *T. khudree* at 37 ± 1°C for 5–10 s [148]. The highest mean motility (83.4 ± 2.1) and fertilization rate (85.6 ± 2.8) was obtained in *C. Idella* when the milt was thawed at 35° C for 30 s [149]. The highest post-thaw motility of 52.6 ± 1.4 s was recorded in *C. carpio* when thawed at 30°C for 30 s [150].

In Salmonid fishes (*O. mykiss, Salmo trutta lacustris, S. trutta fario* and *Salvelinus fontinalis*), the highest fertilization rates obtained was when milt was thawed at 25°C in water bath for 30 s and change of the thawing period for only 5 s or the thawing temperatures for 5°C led to reduce of post-thaw fertilization ability of milt [45]. Cryopreserved milt of *T. khudree* was thawed at 37°C for 40 s in a water bath [120]. Cryopreserved milt of *O. mykiss* was thawed at 25°C in water bath for 30 s for 0.5 ml and 1.8 ml straws and at 60°C for 30s/ 80°C for 20 s for 5 ml straws and was reported that thawing at 25°C in water bath for 30 s was best for thawing of *O. mykiss* milt [42]. The cryopreserved milt of *O. mykiss* was thawed at 10°C for 30s in water bath [130]. The cryopreserved milt of Salmonid fishes was thawed at 25°C for 30 s for 0.5 ml straws and at 30°C for 30 s for 1.2 ml and 5.0 ml straws in a water bath [151].

6. Cryopreservation of fish eggs and embryos

Cryopreservation of fish eggs and embryos are still in its infant stage. Unlike cryopreservation of spermatozoa, very few studies are available on cryopreservation of eggs. Attempts have been made to cryopreserve the eggs of rainbow trout [152, 153] and embryos of Japanese medaka fish, *Oryzias latipes* [154], rainbow trout, *O. mykiss* [155], zebra fish, *Brachydanio rerio* [156], common carp, *C. carpio* [157] and rohu, *L. rohita* [158].

Many of the attempts to cryopreserve fish eggs were failure due to dehydration, relatively large size of eggs, presence of large amount of yolk and different water permeability rate of membranes [159–161]. The major hindrances recorded in the

cryopreservation of egg and embryos of teleost fishes [162] are the large size of fish egg and embryos which results in low surface/volume ratio and lower membrane permeability to water and cryoprotectant solutions that makes the embryos difficult to cool and warm uniformly without damage and ice formation, low permeability of the membrane due to the presence of chorionic layer, sensitivity of fish egg and embryosto low temperatures and the presence of multi-layered membrane structure which hinders the osmotic properties for each compartment of the egg/embryos which finally affects the transport of the cryoprotectant solutions.

Studies have been carried out by different researchers to overcome these issues and some of the efforts made are microinjection of cryoprotectants directly into the cytoplasm [163], use of negative pressure on the egg/embryos to increase permeability of the cryoprotectants [164], microinjection of anti-freeze protein [165] and application of hydrostatic pressure on the egg/embryos [166]. Precise knowledge of embryo permeability is essential for successful cryopreservation of egg/embryos [167].

Herring embryos did not survive after cooling below −10°C when DMSO was used [168]. Methanol was a better cryoprotectant for zebrafish embryo when compared with DMSO or ethanediol since it penetrates theentire embryo within 15 min while other cryoprotectants could not penetrate into yolk even after 2.5 h [169, 170]. Similarly, PG also could not protect the zebrafish embryos upon immersion of it into LN_2 as it resulted in mitochondrial damage, disorganization of ribosomes and plasma membrane of the yolk syncytial layer [171].

7. Application of cryopreservation in aquaculture

- This technology can be used to preserve milt of the best age group brooder which can be used at any point of time in future.
- It can also eliminate inbreeding problem since cryopreserved spermatozoa can be easily exchanged between hatcheries.
- Using this technology, spermatozoa can be made available at any season of the year.
- It makes breeding possible during off-season.
- It synchronizes the gamete availability of both sexes leading to sperm economy.
- It simplifies broodstock management in farms.
- It helps in the production of viable and strong offspring by intra-species hybridization.
- It overcomes the difficulties arising due to the short time viability of gametes.
- It enables the genetic preservation of desired lines.
- It allows cross breeding at different times of the year.
- It helps in germplasm storage for genetic selection programs or conservation of species.

- Cryopreserved spermatozoa can help in the hybridization programmes and genetic engineering research in fishes.

- It leads to many other avenues such as cryobanking of viable gametes as in the case of animal production and development of gene bank and genetic manipulation in fishes.

8. Demerits of frozen milt in aquaculture

- All the milt collected from individuals do not withstand rigors of freezing

- High initial investment cost

- Limits number of sires/males used and if proper care is not taken it may lead to inbreeding

- Requires better training of personnel

- Reduced or poor fertilization rate compared to other artificial breeding methods

9. Conclusion

Cryopreservation technology has been developed for many fish species. However, standard species specific cryopreservation protocols must be developed and the success rate of using cryopreserved sperm in artificial fertilization program of every fish species has to be determined for commercializing the technology. Even though standard protocols of cryopreservation are followed, cryoinjuries are unavoidable. Ways to overcome the cryoinjuries by establishing proper freeze–thaw cycle is essential. The oxidative stress in the cryopreserved sperm must be clearly addressed and methods to reduce the production of reactive oxygen species (ROS) must be evolved. The possible effects of cryopreservation on the energy production, ROS production, mitochondrial DNA of the spermatozoa and the structure of spermatozoa must be documented. Unlike in animals, very few fish sperm banks have been established for fishes. More research is needed to make the sperm banks for fishes a reality in the developing countries. Addressing the research needs mentioned above will help to establish successful fish sperm banks for many commercially important fish species.

Conflict of interest

The authors declare that they have no conflict of interest.

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