CRYOPRESERVATION OF MARINE FISH SPERM- AN EMERGING TOOL FOR ENHANCING FISH PRODUCTION

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Abstract: Cryopreservation of gametes of aquatic species assists the fields of aquaculture and conservation of biodiversity. The increasing demand for aquaculture production has emphasized the need for an efficient and effective means of preserving gametes for better brood stock management, genetic improvement programs and conserving genetic diversity. Cryopreservation has been successfully achieved for fish sperm of many species. The success varied with species and generally better results were obtained with marine species when compared to fresh water species. The major interferences to this technology are technical and technological complications, small sperm volumes, variable results and most importantly, the lack of species wise standardization in the entire process. The paper outlines the general protocol of cryopreservation of marine fish sperm and its futuristic role in increasing aquaculture production.

Keywords: Aquaculture, cryopreservation, fish, sperm.

Introduction: Aquaculture is one of the fastest developing food-producing segments in the world. All through the years, it has been and still expanding and intensifying. The global population is also increasing, thus, it has stressed the demand for aquatic food products. Cryopreservation of gametes has brought creditable changes in reproductive biotechnology contributing significantly to both aquaculture and conservation strategies. It can ensure constant supply and availability of gametes, conserving of endangered and threatened species well as improving the existing to genetic resources [1]-[4]. The foremost studies on cryopreservation of fish sperm was by Blaxter, 1953, [5] and more than 200 fish species have been studied [6],[7]. About 40 of it are marine species [8]. Better results for cryopreservation of sperms are generally obtained with marine species when compared to fresh water species. Major interferences to this process are technical and technological difficulties, small sperm volumes, variable results and most importantly, the lack of species wise standardization in the entire process.

Why sperm and not egg?: The multi-compartmental biological structure, high sensitivity to chilling, low membrane permeability and the larger size limits egg cryopreservation [9].

What is Cryopreservation?: Cryopreservation is the process of preserving biological samples in ultra-low temperatures of -196°C in liquid nitrogen, without losing their viability after following warming to temperatures above 0°C [10], [11]. The technique includes gamete collection, suspending the sperm in an extender, quality analysis, adding of cryoprotectants, equilibration, freezing, thawing, fertilisation and their success assessment [12].

Cryopreservation protocols: Species-specific cryopreservation protocols have been developed for several fishes. Cryopreservation can follow different protocols. It can be a conventional traditional slow method or rapid vitrification process [13]. The slow method results in the formation of ice crystals in the extracellular medium, while no crystals are formed in the rapid vitrification process [14].

The entire process involves the following steps

Collection of Milt from Ripe Males: Milt is collected from ripe males by stripping. Care should be taken to avoid contamination with blood, urine and faeces.

Evaluation of Milt: The milt collected should be evaluated. Colour and nature of milt, milt volume, spermatozoa concentration (Standard Clinical Method [15], viability (Eosine –Nigrosine dye exclusion method [16]), duration and percentage of motility, motility score [17] can be used.

Dilution: Pure semen is usually not suitable for freezing. Milt has to be diluted before cryopreservation. The foremost step in designing a cryopreservation protocol is to select the best composition of the diluent. The commonly used diluents are saline or glucose solution with suitable osmolality containing a cryoprotection agent [4]. An ideal diluent should not trigger sperm motility [7], and should remain sterile and stable during the entire process of cryopreservation. Diluents are used dilute the semen, which in some fish are highly viscous and low volume.

Extenders: Selecting the suitable extender is very important in the cryopreservation process. The efficacy of extenders varies with species. Some extenders may give good results in certain species, while in others it may affect the osmolality of the seminal plasma and sperm motility. Also, the pH of extenders affects the motility of the spermatozoa and fertilization rate of eggs. Salts- sodium chloride, potassium chloride, sugars- monosaccharides or disaccharides can be used depending on the species. Therefore, before using an extender, the osmolality

and pH of the seminal plasma of the particular fish species and the extender have to be studied for obtaining good results. Keeping this in mind simple and effective extender for the fishes can be identified and used.

Cryoprotectant: Cryoprotectants are chemicals that protect cells from damage during the entire process of cryopreservation. Based on the mode of action cryoprotectants are of broadly classified into; permeating and non-permeating. Permeating cryoprotectants as the name suggests are permeable to cells. They enter the cells lower the freezing point of the solution, replace the intracellular water and minimises osmotic shock thereby reducing negative effects of ice crystallization [18]-[20]. The most common permeating cryoprotectants are glycerol, dimethyl sulphoxide (DMSO), methanol, dimethyl acetamide (DMA), ethylene glycon (EG), methyl glycol (2- methoxyethanol), and propylene glycol (PG). Most of these chemicals are lethal and their concentration and equilibrium time can have damaging outcomes and can alter the sperm physiology [2]. Non-permeating cryoprotectants are impermeable to cell membrane, however these compounds are thought to stabilise the plasma membrane during cryopreservation [21]. Sugars, macromolecules like polyvinyl pyrrolidone (PVP) and hydroxyethyl starch (HES) [11], milk proteins, egg yolk, vegetable oils and proteins [22] are the preferred non-permeating cryoprotectants.

Too little as well as too much cryoprotectant is deleterious to the cells [23]. Therefore choice of the cryoprotectant and their optimal concentration and equilibration before freezing is essential for the permeating cryoprotectants to enter the sperm.

Diluent Preparation and Sperm Dilution: Diluent preparation (Extender + Cryoprotectant) and optimizing the semen: diluent ratio is the most crucial step in cryopreservation. The dilution ratios of sperm in extender can vary from 1: 1 to 1: 20 (semen: diluent volume). Lower survival of frozen-thawed spermatozoa was recorded for dilution ratios larger than 1: 20 in Atlantic croaker [24] and larger than 1: 50 in seabream [25]. According to Gwo 1993, the motility of black grouper spermatozoa decreased from 40 to 2 minutes when increasing the semen dilution ratio from 1: 10 to 1: 100 [26]. Increasing the dilution rate from 1: 1 up to 1: 9 did not modify the percentage of motile frozen-thawed turbot spermatozoa [27]. The seminal plasma proteins are known to protect sperm viability but higher dilution ratios greater than 1: 10 may reduce this effect.

Equilibration: The time after diluents are added to semen and before freezing. During this time the cryoprotectants enter the cell as well equilibrates with the surrounding media.

Packing of semen and Freezing in Liquid Nitrogen: Semen is packed either in French straws or Cryovials and plunged into liquid nitrogen at -196^{o} C.

Thawing and Post Thaw Evaluation: The cryopreserved sperm is evaluated after thawing. Thawing is the process of warming the cryopreserved sperm to above temperatures of 0^{o}C. Thawing should be rapid and prevent ice crystallization. Thawed sperm should be used immediately. The post thaw evaluation is carried out to evaluate to success of cryopreservation. Motility and viability are the major points taken into consideration. Artificial insemination can also be carried out if eggs of the species are available.

Slow freezing Protocol: Conventional cryopreservation process make use of cryoprotectants along with a suitable extender that acts as diluent and is a slow freezing method, which can cause dehydration and shrinkage of cells.

Ultrarapid cooling or vitrification : The vitrification method involves direct immersion of small volume samples of semen in liquid nitrogen. However the method requires the use of higher concentration of cryoprotectants. Over traditional cryopreservation technique, vitrification does not require special equipment and is a simple and speedy process [28]. The vitrification device with the sample is plunged directly into the liquid nitrogen so as to achieve a cooling rate of more than 20,000^{o}C/ minute and the entire process can be completed in less than 10 minutes [29]. Direct immersion of samples in liquid nitrogen results in increase in the viscosity of the medium. Thus preventing the water molecules in the sample to become organised to form a crystalline structure [30].

Applications : Cryopreservation of marine fish sperm has numerous benefits. The process can guarantee readiness of gametes, brood stock maintenance and germplasm storage of threatened and aquaculture important species. Sperm cryobanking can help in conservation programs, genetic enhancement of wild and captive populations and in the selection of specific fish features based on their superior reproductive performance. It can also lessen animal breeding costs and serve as cheaper means for inter-continental transport of gametes when compared to live transportation of brood stock. Thus cryopreservation protocol has wide research and application potential.

Limitations: Different factors such as cooling and thawing rates can effect the development of ice crystals, dehydration of cell and cell integrity in the cryopreservation process. Most cryoprotectants can be toxic to the cell and cryoprotectant concentration and equilibration time also have far reaching impact on cryopreservation success. The entire protocol is species-specific. Species-specific protocol is tedious

and time-consuming process and also collecting the sperm in the correct and viable state is also not easy.

Conclusion

Numerous studies and research have been accomplished through the years to design cryopreservation protocol for marine fish sperm. The success of this cryopreservation process can aid artificial fertilization protocols. More investigation and focus is required in this regard to develop a streamlined technology for field level application of this technology. Enhancement in these areas no doubt would contribute to assured success regarding the use of cryopreserved fish sperm for aquaculture.

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