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Director, CMFRI, Kochi

Dr. P.C. Thomas
Winter School Director

Co-ordinators:

Dr. R. Paul Raj, Head, PNPD
Dr. K.C. George, Principal Scientist
Dr. P. Jayasankar, Senior Scientist
Dr. D. Noble, Senior Scientist

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Course Manual

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INDIAN COUNCIL OF AGRICULTURAL RESEARCH
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
P.B. No. 1603, Tatapuram P.O.,
Kochi – 682 014

CRYOPRESERVATION OF MARINE FISH GAMETES FOR MARICULTURE APPLICATIONS

Dr. D. Noble
Central Marine Fisheries Research Institute, Cochin

Introduction

Gamete cryopreservation is a wide and complex topic. It is not confined to Cryobiology alone, but also encompasses into complementary disciplines like Reproductive Biology, Cell Physiology and so on. Cryopreservation of gametes also covers invertebrate and vertebrate species and it addresses various cell and tissue types, more complex sperm aggregates called spermatophores and multifunctional tissues like eggs and embryos. While cryopreservation of eggs and embryos is still at stage of basic research, spermatozoa can be successfully cryopreserved for most species and in particular for several teleost fish, the method has already reached a standard that makes it interesting for routine and commercial use.

The importance of cryopreservation steadily increases for purposes of fish culture and conservation of biodiversity. Compared to fresh water species, a high percentage of marine fish spermatozoa survive cryopreservation, as a consequence, the success in breeding is greatly enhanced by the use frozen sperm. Because of the simplicity of the technique, cryopreservation of sperm in marine species is suited for large scale application in Mariculture. As marine fish farming expands, there is an increasing need to apply sperm cryopreservation techniques.

The benefits of this technique include: (1) synchronization of gamete availability of both sexes, (2) use of total volume of milt available, (3) simplicity of broodstock maintenance, (4) transport of gamete, (5) avoiding aging of the sperm,. (6) experimental programmes, (7) conserving genetic variability and (8) protection of endangered species. In protogynous hermaphrodite species, such as black grouper *Epinephelus malabaricus,* sperm can only be collected in inverted males 5-10 years old and success in breeding is greatly enhanced by the use of cryopreserved spermatozoa. Of particular interest to marine biotechnologists is in genetic studies, development of hybrids, clones, polyploids and disease resistant strains.

Principles of cryopreservation

Around 0°C, spermatozoa can be stored for few hours to several days, while cryopreserved gametes can theoretically be stored indefinitely without much deleterious effect. Storage temperatures below −130°C, all biological molecules become motionless and therefore, cannot participate in any biochemical reaction and hence the metabolic activity is arrested. A commonly used storage temperature is −196°C (temp. of liquid nitrogen) simply because liquid nitrogen is a convenient and widely used storage medium.

The freezing and thawing of biological material involves a series of complex and dynamic physico-chemical process of heat, water and ionic transport between cells and their surrounding medium. A knowledge of the biology, including the ultra structure of fish spermatozoa is important for understanding and designing of preservation procedures for the gametes. Most of the fish species which have been subjects for sperm cryopreservation, are all external fertilizing teleosts with anacrosomal aquasperm. Cryoinjures during cryopreservation procedure often occur. The success of cryopreservation depends on minimizing the extent of cryoinjures during freezing and thawing.

CRYOPRESERVATION PROTOCOL

I. Milt collection

Milt is a suspension of spermatozoa in the seminal fluid. Sperms of fishes are immotile when they are within the testis and in undiluted form. Activity is only initiated by dilution with water/saline or ovarian fluid. Several factors may be responsible for the immotility but not clearly understood, possibly O2 depletion, a protein androgammone? pH, or KCl con. (more of K+ inhibits and Na+ activate). In general, spermatozoa of fresh water and saline water spawners are activated by hypotonicity and hypertonicity respectively of their surrounding media. Motility is considerably shorter in fresh water spawners because of osmotic injury. There is positive correlation between motility and fertility and motility is often used as an indicator of fertility in artificial insemination, because it is easy to assess microscopically. Sperms taken from properly stored dead fish are viable for 1½ to 5 hours.

Oozing male fish can be stripped manually by applying gentle pressure on the abdomen. All aseptic precaution should be taken and also care should be exercised to avoid contamination with blood, urine, scales and faeces. An intramuscular/intraperitoneal injection of 'Ovaprim' 3ml/kg body weight can be given 8 hours prior to stripping.

II. Evaluation of milt

Evaluation of milt quality is done by placing a drop of milt in a glass slide and its motility checked in sea water. A cover slip is carefully placed over it and observed the motility of sperms at the inter-junction of milt and sea water in a compound microscope at a magnification of 10x. Three main types of movement could be observed: (1) rapid, progressive or shooting movement, (2) sluggish or lethargic movement and (3) vibration *in loco*. A quick estimation of the approximate percentage of spermatozoa belonging to each category can be made and accordingly a motility score (1–5) can be given. Only samples with a motility score of 3 and above are fit for cryopreservation.

Criteria for motility score

Criteria	Score
90% or above of the sperms exhibiting rapid progressive or shooting movement	5
75% or more exhibiting rapid progressive, 10% sluggish and the rest immotile	4
50% exhibiting rapid progressive, 25% sluggish and 10% vibrating *in loco* and the rest immotile	3
25% exhibiting shooting movement, 50% moving sluggishly, 10% vibrating *in loco* and the rest immotile	2
occasional sperm shooting, 10% showing sluggish movement, 50% vibrating *in loco* and the rest immotile	1
completely immotile	0

III. Dilution

a. Extender

Milt has to be diluted before cyopreservation. A suitable extender containing a cryoprotectant may be used for this purpose. An extender is a solution of balanced salts and some times organic compounds mimicking the composition and osmolality of seminal plasma. One of the functions of the salt or organic compounds is to inhibit osmotically the activation of the sperm motility. As motility of sperm depends on internally stored ATP which can be resynthesised only at very slow rates, the extender must ideally inhibit sperm motility before freezing since fertility declines after activation probably due to sperm exhaustion.

Composition of some of the commonly used extenders

Extender Composition (mg)	CC1 (Kurokura *et al,* 1984)	Rana and Mc Andrew (1989)	Chao (1975)	Marine teleost ringer solution	Mixture B(Elizabeth, 1987)	Mixture C(Elizabeth, 1987)	V_2E (Scott& Baynes, 1980)
NaCl	750	650	1350	1350	600	600	750
KCl	20	300	60	60	38	38	38
$CaCl_2$	20	30	-	-	-	23	-
$NaHCO_3$	20	20	20	20	200	100	200
$NaHPO_4$	-	-	-	-	-	41	-
$MgSO_4$	-	-	35	35	23	23	-
$MgCl_2$	-	-	-	-	-	-	-
Na_2HPO_4	-	-	-	-	53	-	-
Glucose	-	-	5000	-	-	-	100
Egg yolk	-	-	-	-	-	-	20
Water	100	100	100	100	100	100	100
pH	7.3	7.3	7.2	6.8	7.0	7.3	7.0

b. Cryoprotectant

Unprotected cells can survive freezing if they are cooled at optimal rates, but generally cooling rate low enough to prevent intracellular freezing is low enough to produce lethal exposure to high concentrations of electrolytes. The major role of cryoprotectants is to bind electrolyte and thereby preventing these substances from concentrating in the residual unfrozen solution in and around cells during freezing. Additionally they bind with water molecules and reduce pure crystal formation. They also lower freezing point of intracellular fluid to –45°C. To be effective, a cryoprotectant must permeate into the cell, otherwise it would not prevent a rise in intracellular electrolytes during freezing. However, some macromolecules which cannot enter cells have been shown to protect against freezing damage.

Cryoprotectants can suppress most of the cryoinjuries, but when used at higher concentrations most of them become toxic to biological materials. Their toxicity should be less than their protective ability or they would damage rather than protect the cells. Choice of cryoprotectant is normally on the basis of toxicity and permeability to cells and solubility in water during freezing. Though the optimum concentration may vary between cryoprotectants, species and equilibration time used, a final concentration between 7 and 15% have been successfully used in most cases. Cryoprotectants can be divided into two groups – those permeable to cell membrane and those not permeable.

(i) Permeating cryoprotectants

Commonly used permeating cryoprotectants are dimethyl sulphoxide (DMSO), glycerol, methanol and 1,2 – propanediol. Of these compounds, the glycerol is the least toxic to most biological material but also the least permeable to cell membrane and hence takes longer to equate with glycerol osmolality. Methanol on the other hand is highly permeable to cell membranes, but it is generally considered most toxic. DMSO is fairly permeable to membranes but its toxicity intermediate between glycerol and methanol and can be minimized by reducing the temperature. For this reasons, DMSO is the most widely used permeating cryoprotectant.

Concentration of cyoprotectants used

Sl. No.	Cryoprotectants	Concentration (%)
1	DMSO	7.5, 10, 12.5
2	Glycerol	5, 10
3	Methanol	5, 10
4	Propylene glycol	8, 12
	Combinations:	
5	DMSO and Glycerol	5+5
6	Methanol and Glycerol	5+5
7	DMSO and Propylene glycol	5+5
8	Methanol and Propylene glycol	5+5

(ii) Non-permeating cryoprotectant

Non-permeating cryoprotectants include sugars (glucose, sucrose), polymers (polyvinyl pyrolidone) and proteins (egg yolk, serum proteins, skimmed milk). Their protective ability usually in conjunction with a permeating cryoprotectant, is related to their ability to depress freezing point and prevent ice crystal formation. Specific lipids with potential for lowering membrane phase transition temperature are used to minimize membrane damage during initial cooling and during freezing.

The milt is generally mixed with cryodiluent in the ratio of 1:3. All solutions are to be maintained at 20°C.

IV. Equilibration time

Equilibration time is the time after adding the diluents (extender+cryoprotectant) and before freezing. During this period the cryoprotectant penetrates the cells and equilibrates with the surrounding media. Fish spermatozoa may become motile upon mixing with diluents, hence the equilibration time is kept minimum to avoid sperm exhaustion. This precaution will in turn, minimize cryoprotectant toxicity. As spermatozoa are sufficiently small and DMSO penetration is rapid, no lengthy equilibration period is required for this cryoprotectant. However, penetration of glycerol is slow and a longer equilibration is required. By vacuum equilibration technique equilibration time can be shortened greatly by augmenting penetration of cryoprotectant, at the same time minimizing toxicity. With the exception of glycerol, permeability of most cryoprotectants is not markedly reduced by low temperatures and therefore, equilibration is usually performed at 0°C to reduce cryoprotectant toxicity. Exposure to glycerol is preferably done at room temperature.

Equilibration time of 10 mts over ice is to be given including the time to fill the diluted milt into 0.5 ml French straws. Fill the straws and seal with polyvinyl alcohol powder.

V. Freezing and storage

Milt prepared from previous phase is cooled from 0 to –196°C during this phase. It should be noted that even with the use of protective agents, there is still an optimum cooling rate, though a very wide one for fish spermatozoa. For freezing of fish spermatozoa, a two step procedure is generally applied – milt is cooled in liquid nitrogen(Ln) vapour on a floating tray or hanging in the in the neck of the container and then straws are plunged into the Ln. The cooling rate is determined by the height of the tray or the depth at which the straws are lowered in Ln. The cooling rate successfully used is 30–160°C/minute.

Storage temperature is usually –196°C in Ln. Maintenance of this temperature is essential and spermatozoa can be stored indefinitely without appreciable deterioration.

VI. Thawing

At this phase gametes are warmed from storage temperature to above O°C. Rapid thawing is necessary to avoid recrystalization. Thawing rates in marine fish are 10–40°C/minute. Thawed spermatozoa must be used immediately. Generally the motility rate of freeze–thawed spermatozoa is high in marine fish species compared to fresh water species. In marine species, the high motility rate of frozen-thawed spermatozoa also results in high fertilization rate. The duration of motility in post–thawed sperm markedly reduces. Cryoinjuries have been reported in thawed spermatozoa of many fish species, but relatively less in marine fish. The better ability to withstand the rigors of cryopreservation may be due to the lipid composition of sperm membranes, mainly the molar ratio of cholesterol to phospholipids which is two or three times higher than the freshwater fish.

VII. Post–thaw evaluation

The motility of frozen thawed semen is a reliable parameter for quality determination, since there is statistically greater correlation with post–thaw fertilization rate. Post-thaw motility assessment can be carried out by rapidly plunging the straws into water bath at 37°C for 20 sec. The sealed ends of the straws are cut open to expel the thawed milt. A small drop of thawed milt taken on the glass slide and mix with seawater and immediately observe under the microscope(10x). Post-thaw motility can be judged by two variables – (a) percentage of motile spermatozoa on a 5 point scale (b) duration of motility using a count up timer.

After thawing, spermatozoa may show different motility characteristics from untreated sperm. The duration of motility can be markedly reduced from few minutes to few seconds. Only an empirical quantification of actual fertilization is a reliable indicator of success of cryopreservation procedure.

Artificial insemination & *in vitro* fertilization

Gametes that have survived the cryopreservation procedure are ready for artificial insemination. Artificial insemination has to be performed immediately after thawing, since the delay will reduce the fertility rate. The speed of sperm movement depends on temperature. Since the energy reserve of fish spermatozoa is limited, the increased speed caused by rise in temperature shortens the life span. The lowest limit for natural spawning (0-10°C) is generally used as the temperature for insemination.

An insemination medium has to be given for adequate distribution of spermatozoa in relation to ova. Freshwater or seawater are not the best medium for freshwater or marine fish sperm respectively as morphological changes takes place in these media. Insemination media best adapted to dilution of fresh and marine fish sperm have a salinity of 4-7% and 20% respectively and pH value about 9 in both types. In these solutions sperm injuries are minimal.

The best fertility of fish spermatozoa diluted in insemination medium can be obtained at an optimal sperm concentration and fertility declines more rapidly when the dilution exceeds 1:10 (v/v). Not all spermatozoa survive cryopreservation procedure and densities of frozen-thawed sperm have to be higher than for fresh sperm to maintain the same level of fertility.

Conclusion

Simple cryopreservation protocols are available for marine fish spermatozoa. The extender generally consists of saline or sugar solution in which DMSO added as cyoprotectant. Methodologies such as exposure of straws on tray in the vapour, immersion in Ln and thawing in water bath are used for freezing and thawing. To date, the semen of about 30 different marine fish species have been cryopreserved and a high survival of frozen-thawed spermatozoa is often recorded. Work on marine species have mostly been concentrated on the improvement in freezing technique protocols, but lacked studies involving morphological and metabolic changes. Also, the quality of sperm sample before freezing should be investigated in more detail, as the problem of semen aging and urine contamination can alter the biological features of the spermatozoa and their suitability for freezing. The possible improvement in sperm fitness for cyopreservation by modifying rearing parameters during spermatogenesis (water temperature/food composition) has not yet studied in marine fish species.

The cryopreservation technique for spermatozoa of marine fish is applicable for production purposes in aquaculture, as well as for establishment of gene banks. Coupled with insemination, cryopreservation will lead to an improvement of gamete management in Mariculture.

Suggested reading

1. Jamieson, B.G.M. (1991) Fish Evolution and Systematic Evidence from Spermatozoa. Cambridge University Press

2. [illegible], R.W. and S.J. de Groot (2000) Cryopreservation of gametes in Aquatic Species. Aquaculture Research (Special Issue), Volume 31, Number 3, March 2000.