REVIEW PAPER

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(Accepted 23 December 1996)

Journal of TFRI TAIWAN 1993 Fisheries Research

Cryopreservation of Finfish and Shellfish Sperms

Abstract

Cryopreservation of finfish and shellfish sperm has a longer history than that of eggs and embryos hence with a fruitful result. The first part of this paper introduces the principles of cryopreservation including cryofreezing, thawing and cryogenic storage. The second part describes five major injuries related to freezing and thawing in the generalized cryopreservation procedures. They are pH fluctuation, cold shock, ice crystal formation, permeability and toxicity of cryoprotectants. The third part discusses the basic steps affecting sperm survival in the series of freezing procedures and compares for those successful finfish and shellfish species in Taiwan. The steps are (1) collection and assessment of sperm, (2) equilibration of cells in extender and cryoprotective agents, (3) freezing of sperm suspension using controlled or uncontrolled cooling rates, (4) cryogenical storage at -196°C, and (5) thawing of frozen sperm for fertilization.

Key words: Finfish, Shellfish, Sperm Cryopreservation

Cryopreservation of eggs and embryos of both finfishes and shellfishes has a rather short history with very limited results. Successful cryopreservation of sperm, with either understanding of basic principles and improvement of related technologies, or development of replicable practices, has been reported in many species of finfishes and several species of shellfishes[1-7].

In general, low temperature preservation is a pretreatment of experimental material, followed by a short-term storage at 4°C, while cryopreservation involves a short- or long-term storage of treated material, using liquid nitrogen as a coolant. Cryopreservation may offer the supply of gametes of certain species of aquatic organisms and help maintain the genes of endangered species. The species under cryopreservation study are mainly those of commercial importance and have an advantage of availability of gametes[8].

Chao, N. H. (1996) Cryopreservation of finfish and shellfish sperms. J. Taiwan Fish. Res., **4**(2): 157-170.

The potential applications of cryopreservation of gametes in finfishes and shellfishes are as follows:

1. To guarantee commercial importance by availability at any time and place.
2. To avoid losses caused by diseases.
3. To offer appropriate usage in hatchery and laboratory on a continuum basis.
4. To provide convenience in transport of broodstocks between hatcheries and farms.
5. To improve possibility of crossbreeding.
6. To expand the range of using finfishes and shellfishes as experimental broodstocks.

In this paper, an overview of the principles of cryopreservation, cryoinjuries, factors of affecting cryopreservation, and short- and long-term cryopreservation of fish sperm are presented.

Principles of Cryopreservation

Cryopreservation may be accomplished by storage

of living cells below -135°C, at which there is no growth of ice crystals, and rates of biophysical processes are too slow to affect cell survival. There is series of complex and dynamic physico-chemical processes of heat and water transport between treated cells and their surrounding medium in the processes of freezing and thawing in cryopreservation.

I. Cryofreezing

When cells of aquatic organisms are cooled in an aqueous solution and both cells and solution are supercooled, heterogenous nucleation takes place in the extracellular solution. When it occurs intracellularly, the resultant nuclei are isolated by plasma membranes from other unfrozen cells. When water is frozen, the extracellular solution becomes progressively concentrated. In the case of low cooling rate, the cells have a plenty of time to lose water, so that they remain in the state of osmotic equilibrium with the gradually concentrating extracellular solution. A prolonged exposure to concentrated solution is lethal. In the case of high cooling rate, they have insufficient time to diffuse water out of the cells to become ice crystals. Intracellular freezing is generously fatal. In the case of extraordinary small microorganism cells, all the water in the cells can be withdrawn in the freezing process, so that they are effectively desiccated. In the case of a sufficiently high cooling rate, that minimizes the time of exposure to concentrated solution and also keeps the amount of intracellular ice at a safe level, a balanced situation that are achieved allows the survival of the cells. There are chemicals that increase the balance of the effects between intracellular ice and extracellular concentrated solution, for improving cell survival. They are cryoprotectants or cryoprotective agents (CPA).

If values of factors required for mathematical modelings of cryopreservation are known, the optimum cooling rate can be estimated[9-11]. The factors are:

1. The viscosity of intracellular solution.
2. Surface to volume ratio of cell.
3. Rate of diffusion of water through cell membrane.
4. Distance between cell and nearest ice crystal.
5. Viscosity of extracellular solution.
6. Permeability of CPA added to cell solution.

Theoretically, an extremely high cooling rate allows cell survival, if the thawing rate is extremely high. When it happens, intracellular nuclei may form but they have insufficient time to grow, or glass transformation may occur with little nucleus formation.

In general, the size of a cell (surface area to volume ratio) and its permeability to water determine how quickly water can escape and how slowly the cell must be frozen. Too slow a cooling rate leads to over-dehydration of the cell with excessive concentration of solutes, resulting in the so-called solution effect damage. Whereas, too rapid the rate leads to inadequate dehydration with subsequent formation of a large amount of intracellular ice crystals which are lethal to most of the living cells.

II. Thawing

The biophysical processes occurring in freezing cells are reversed when the frozen cells are thawed. The rate of water flowing into the cells depends upon the warming rate and permeability of the cells to water. If thawing is not fast enough, some unbound water in the cells is recrystallized. The ice crystals are liable to disrupt intracellular organelles and membranes, resulting in death of the cells. Schneider[12] reported that a rapid change in solute gradients causes a membrane damage to mouse embryos in rapid thawing.

III. Cryogenic storage

Desirable cryogenic temperature is -130°C or lower, and -196°C of liquid nitrogen is most

popularly used, because it is readily available, convenient and inexpensive. Biological molecules are motionless and do not participate in biochemical reactions at the cryogenic temperature. Theoretically, biological material can be stored indefinitely in the frozen state, at this temperature although biochemical reactions at the atomic level may still take place. DNA damage caused by background radiation is cumulative, because no DNA repair may take place at -196°C. According to Glenister and Lyon[13] mouse embryos exposed to an equivalent of 2,000 years of background radiation show no observable deterioration. The regression equations derived from actually measured yields help estimate that human red blood cells can becryopreserved for 1.4 years at -80°C and 46.7 years at -196°C with the recovery rates higher than 80%. Finfish and shellfish sperms are able to store for at least three years which are long enough for most of the cryopreservation purposes.

Cryoinjuries

The major injuries related to freezing and thawing processees within the temperature ranges of generalized cryopreservation procedures are listed in Fig. 1. Most of the cryoinjuries take place over the temperature range betwean 0 and -40℃ due to two major causes : heat removal and application of cryoprotectants.

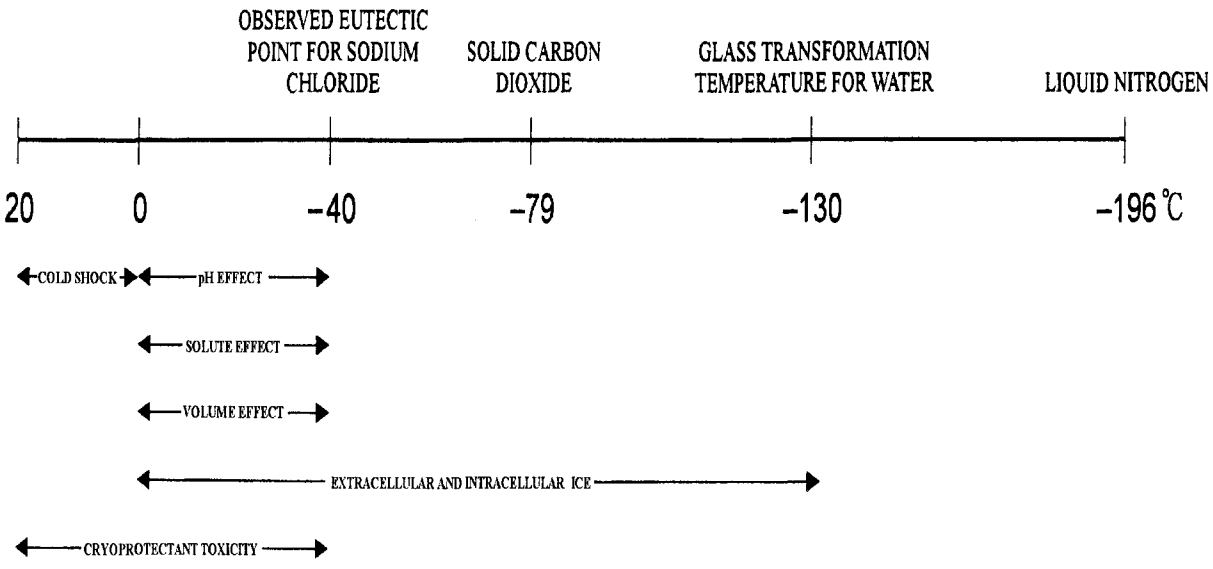

Fig. 1. Major cryoinjuries related to temperature changes (after Leung and Jamieson[6]).

I. pH fluctuation

Most biological salts are eutectic at a temperature range of 0 to -55°C. In cryofreezing and thawing, the buffering function of these salts is destroyed and pH of the biological solution changes. The pH fluctuation may be caused by freezing or addition of cryoprotectants.

II. Cold shock

Cold shock is caused by change in membrane lipids from the liquid phase to the solid phase in the freezing process from 10 to -16℃. Low temperature alone may not be sufficient for the phase change. At this temperature range ice crystals spontaneously form to dehydrate lipids, that undergo change from lamellar liquid crystals to gel phase transition. Cold shock may be caused by increase in membrane tension resulting from temperature reduction.

III. Ice crystal formation

Both ice crystal formation and solute concentration contribute to cell damage in the freezing process. If solute concentration alone caused cell damage, a very rapid rate of cooling would be an ideal for the freezing process, since there would be insufficient time for water to leave the cells. In contrast, if intracellular ice damage were the sole contribution factor to the cell damage, a very slow rate of cooling would be most ideal, because this would provide the maximum time for water to leave the cells. Because both solute concentration and intracellular ice formation contribute to the cell damage, an ideal cooling rate should be neither too fast nor too slow.

IV. Permeability

Cell permeability plays a major role in cryoinjuries. Less water is removed from cells that are less permeable in the freezing process. The more permeable cells demonstrate a greater tolerance to the cooling and freezing processes, but they appear to be dehydrated. In an aqueous suspension of living cells, ice crystals are formed first in the solution surrounding the cells, resulting in increasing concentrations of solutes outside the cells. Because of the difference in osmotic pressure between inside and outside the cells, water continues to move out the cells as long as the inbalance in salt concentrations remains. The cells are drying as freezing proceeds extracellularly, as rate and extent of this process depend upon the rate of cooling and permeability of the cells.

V. Toxicity of cryoprotectant

High water solubility and low toxicity to cells are two essential considerations for a chemical to be cryoprotective. It must be non or less toxic, able to penetrate cell membranes easily, and able to bind either electrolytes to increase concentration in the freezing process or water molecules to delay freezing. The toxicity is a function of kind and stage of gametes to be frozen. In cryopreservation, it is impossible without CPAs, but its toxicity may cause gamete mortality in pretreatment and post-thawing. It is essential to determine the point of equilibrium between cryoprotective efficiency of cryoprotectants and toxicity tolerance of experimental species in the future researches on cryopreservation.

Cryopreservation of finfish and shellfish sperms

The species of finfishes and shellfishes listed in Tables 1 and 2 have been the subjects of sperm cryopreservation studies. They are of commercial importance in Taiwan.

Basic steps in the freezing procedure that affect sperm survival are considered for each species and compared among species, based on the experiments conducted at the Taiwan Fisheries Research Institute in recent years.

I. Collection and assessment of sperm

The age of maturation of experimental finfish species varies from one species to the other, such as 10 months for tilapia *Tilapia zilli,* 1 to 1.5 years for black porgy *Acanthopagrus schlegeli* which is protandric, 2 to 3 years for grey mullet *Mugil cephalus,* 5 to 8 years for milkfish *Chanos chanos,* at

least 5 years for grouper *Epinephelus malabaricus* which is protogynic, 10 months for small abalone *Haliotis diversicolor*, and 6-10 months for Pacific oyster *Crassostrea gigas* and hard clam *Meretrix lusoria*. Aging of sperm in vivo has been identified as a cause that reduces its storage life at 4℃. Surival of fish sperm can be maintained at 0-4℃ longer by providing adequate air or oxygen to the milt sample[14]. Preservable duration of sperm decreases as the spawning season goes by.

Table 1. Finfish species used in current sperm cryopreservation studies in Taiwan.

Species	Experiments on				Proof of motility after preservation		Fertility test in		Fertilization rate (%)*	
	Sperm characteristics	Extender selection	Optimal cryoprotectant	Freezing rate	Short term	Long term	Laboratory	Hatchery	Satisfactory frozen milt	Control fresh milt
Mugil cephalus	○	○	○	○	○	○	○	○	64.9 38.9 48.9	23.6 47.4 38.4
Acanthopagrus schlegeli	○	○	○	○	○	○	○	○	91.5 77.4 99.0 71.4	96.0 62.3 98.0 84.5
Epinephelus malabaricus	○	○	○	○	○	○	○	○	91.5 77.4 99.0 71.4	96.0 62.3 98.0 84.5
Oreochromis aureus	○	○	○	○	○	–	–	–		
O. mossambicus	○	○	○	○	○	○	–	–		
O. niloticus	○	○	○	○	○	–	○	○		
O. niloticus × *O. aureus*	○	○	○	○	○	○	○	–	72.7	85.7
Oreochromis sp.	○	○	○	○	○	○	○	–	93.4	96.0
Tilapia zillii	○	○	○	○	–	○	○	–		
Chanos chanos	○	○	○	○	○	–	–	–		
Siganus oramin	○	○	○	○	○	○	–	–		
Plecoglossus altivelis	○	○	○	○	○	–	–	–		
Lateolabrax japonicus	○	○	○	○	○	–	–	–		
Misgurnus anguillicaudatus	○	○	○	○	–	–	–	–		
Boleophthalmus chinensis	○	○	○	–	○	–	–	–		
Micropterus salmoides	○	○	○	○	–	–	–	–		

*Only samples with satisfactory results are shown.

Table 2. Shellfishes used in current sperm cryopreservation studies in Taiwan.

Species	*Experiments on*		*Proof of motility after preservation*		*Fertilization rate (%)*	
	Optimum cryoprotectant	*Freezing rate*	*Short term*	*Long term*	*Frozen milt*	*Fresh milt*
Small abalone (*Haliotis diversicolor*)	○	○	○	○	94.80 89.10	98.80
Pacific oyster (*Crassostrea gigas*)	○	○	○	–	28.00 37.30	82.00
Hard clam (*Meretrix lusoria*)	○	–	○	–	76.00	78.00

Sperm of black porgy collected in the early season can be stored at 4℃ longer than that in the late season[15]. Similar result was found for European bass (*Dicentrarchus labrax*)[16]. For the above species, the seasons for sperm collection recommended are as follows: February to July for tilapia, December to February for black porgy, December to January for grey mullet, May and September to October for milkfish, and April to July for grouper. Regarding to shellfishes, September to November is for small abalone, January to September for Pacific oyster, and May to August for hard clam. Generally, temperature induction is adopted to allow small abalone and hard clam spawners to expel milt into water for convenience of the experiments.

In the hermaphroditic protandric black porgy cultured in pond, it is easy to collect large amounts of milt from the harvested fish which are 1.5 to 2 years old. In addition to stripping the male spawners to obtain sperm, slicing testes of postmotem fish (within 10h postmortem) to obtain sperm suspension or inserting a microcapillary tube into the genital pore are two effective methods for grouper that has scarce milt[17]. In order to maintain the quality of sperm, the following attentions should be taken:

(a) Collecting the sperm without contamination of fish feces, blood or scales.

(b) Holding the fish for collecting sperm at the best condition by providing moisturized air for respiration. For example, the one third of the container is filled with milt and the two thirds with humidified air.

(c) Maintaining the temperature for collected milt at 4℃ in the field and transportation. It was found that the collected sperm of postmortem *E. malabaricus* retains its viability for cryopreservaton after being stored at 4℃ for several hours.

For both finfishes and shellfishes, motility of sperm is assesed by using microscope and a conventional standard as listed in Table 3 or by using a motility analyzer. The analyzer consists of an automatic analytic sequence with internal optical image assembly, a microprocessor system for image analysis (30 photorecords per second), an internal printer, and a color monitor showing track velocity, progressive velocity, path velocity and straightness.

II. Equilibration of cells with extender and cryoprotective agents

In most of the early phase of the studies concerned with cryoprotective agents, rather complicated formulae, such as Ott and Horton extender 164 (sodium citrate, glucose, latcose

of various concentrations), were adopted to prepare diluents[18-19]. The effects of various concentrations of several important agents on sperm motility at the pre-freezing stage were investigated. However, the effort was not successful. The preparation was complicated, took much time and not beneficial. Later on, the diluent of simple and single agent, such as 0.5% honey or 5% glucose in the Ringer solution, was found very effective in both laboratory and field for milkfish and black porgy sperm[20].

Table 3. Categorization of fish sperm motility under microscope reading. (after 21)

	Value	*Observation*
	5/+++++	All sperm moving vigorously ; almost impossible to fix vision on any one spermatozoa
	4/++++	Some sperm moving slowly enough to see easily ; most still whirling or swimming vigorously
	3/+++	Some sperm still ; some sperm slowly swimming ; some fast
	2/++	Very few fast swimmer ; many still ; some slow "lazy-looking" sperm
	1/+	Only one or two sperm swimming in each field
Minimum	0/–	No sperm moving ; Brownian movement the only detectable motion

Milk diluted with the Ringer solution and methanol is an ideal freezing medium for cryopreserving sperm of several tilapia species, including *Oreochromis aureus, O. mossambicus, Tilapia zilli, O. niloticus, O. aureus* hybrid and red tilapia *Oreochromis* sp. hybrid. Without milk the frozen-thawed sperm does not function because of the unusually high stickness of the milt mixture in which spermatozoa are immobile. The compositions of several diluents of extenders are shown in Table 4.

Table 4. Comparison of diluents of extenders used for cryopreservation of sperm in various fish species.

Ingredient (g/l)	*NaCl*	*KCl*	*$CaCl_2$*	*$MgCl_2$*	*$NaHCO_3$*	*Taps*	*Caps*	*Glucose*	*Yolk*	*Honey*	*Milk*	*Species*
Marine fish ringer	13.5	0.60	0.25	0.35	0.2	–	–	–	–	–	–	Marine fish
Freshwater fish ringer	7.5	0.2	0.20	–	0.2	–	–	–	–	–	–	Freshwater fish
Taps	2.9	3.20	0.07	0.03	–	15 mmol	–	–	–	–	–	Tilapia
Caps	2.9	3.20	0.07	0.03	–	–	15 mmol	–	–	–	–	Tilapia
Milk in ringer	7.5	0.20	0.20	–	0.2	–	–	–	–	–	150 ml	Tilapia
V_2e	7.5	0.38	–	–	2.0	–	–	1.0	0.2	–	–	Tilapia
V_2f	7.5	–	–	–	2.0	–	–	1.0	0.2	–	–	Tilapia
Honey in ringer	13.5	0.60	0.60	0.35	0.2	–	–	–	–	1.0 ml	–	Black porgy; milkfish

Recently, it has been found that adding a cell culture medium, Menezo B2 Medium INRA (15%), to the glucose and Ringer solution is a very effective method of improving the pre- and post-freezing sperm motility of grouper[17]. A similar observation was also recorded for common carp *Cyprinus carpio* by Cognie et al[22]. Several other cell culture media have been also suggested to be tested for more finfish species. The acceptable dilution ratio is, 1 part milt: 1 part extender for sperms of grey mullet, black porgy and tilapia, 1:4 for milkfish, and 1:20 for grouper.

CPAs provide cryopreservation a labile enzyme (catalase) and stabilize protein in unfrozen aqueous solution. A wide variety of chemicals share this property, but only ethylene glycerol, methanol and dimethylsulfoxide (DMSO) have been used in the studies on cryopreservation of finfish and shellfish sperms.

CPAs may induce protein denaturation at higher temperature, and thus are toxic to cells. Cryoprotectants at higher concentrations can prevent the ice formation and concomitant freezing-thawing damage of cells. However, cryoprotectants at the same level can be lethal to unfrozen cells. Toxicity of CPAs is a factor affecting the success of cryopreservation of sensitive cells such as sperm. For each experimental species, effects of CPAs at various concentrations on pre-freezing motility of sperm have been studied.

In the absence of CPAs, very few spermatozoa survive freezing in cryopreservation. Addition of CPAs to milt greatly extends the tolerance of spermatozoa to freezing, when the freezing rate is slow. It has been reported that the optimal cooling rate depends on nature and concentration of cryoprotectant used. In grey mullet and black porgy , 5 to 15% glycerol function well, while in tilapias 15% milk and 5% methanol as diluent-CPA mixture ensure satisfactory sperm motility and fertility. On the other hand, for milkfish and grouper, a high proportion of sperms in 10% DMSO retains their motility. Table 5 is a list of CPAs used in the cryopreservation studies for fishes and nonfishes.

Table 5. Cryoprotectants used in cryopreservation studies.

Acetamide	Glycerol monoacetate	Proline
L. Alanine	Glycine	Propylene giycol
Albumin	Hydroxyethyl starch*	Pyridine-N-Oxide
Ammonium acetate	Inositol	Ribose
Chloroform	Lactose	Serine
Choline	Magnesium chloride	Sodium bromide
Dextrans*	Magnesium sulphate	Sodium chloride
Diethylene glycol	Maltose	Sodium iodide
Dimethyl acetamide	Mannitol	Sodium nitrate
Dimethyl formamide	Mannose	Sodium sulphate
Dimethyl sulphoxide (DMSO)	Methanol*[a]	Sorbitol
Erythritol	Methyl acetamide	Sucrose*
Ethanol	Methyl formamide	Triethylene glycol
Ethylene glycol*[a]	Methyl ureas	Trimethylamine acetate
Formamide	Phenol	Urea
Glucose	Pluronic polyols	Valine
Glycerol*[a]	Polyethylene glycol*[a]	Xylose
−glycerophosphate	Polyvinyl pyrrolidone*	

* This cryoadditive confers greater protection than others listed here.

[a] Used in cryopreservation of fish spermatozoa.

In general, a majority of researchers working on sperm-freezing of teleosts agree in using either glycerol or DMSO with concentrations between 3.3 and 15% (v/v) as the cryoprotective agent[21-23]. In our studies, methanol was used only in tilapia[24] similar to that for zebar fish by Harvey et al.[25]. Both DMSO and glycerol at concentrations up to 20% (v/v) stimulate RNA synthesis[9]. The stimulation is due to an increase in initiation with the DNA template rather than the RNA polymerase.

III. Cooling rate for freezing sperm suspension

The cooling rate is critical for cryopreservation. When sperm is cooled too rapidly, intracellular ice crystals are formed. To prevent the formation of intracellular ice, the sperm must be cooled slowly. For sperms of certain species in the presence of certain CPAs, there is a critical subzero temperature zone. Within this zone, the sperm is exposed to increasing concentrations of solutes, and therefore, should pass this zone as quickly as possible. In contrast, within the same temperature range the sperm requires a sufficient time to get rid of water, so that ice is not formed, and the sperm can stand cooling to lower temperature.

In reconciliation of the above two factors, the optimal cooling rate is determined for different cryoprotectants. The temperature at which spermatozoa can be plunged directly into liquid nitrogen without causing a reduction in motility should be carefully determined. One of the consequences of the two-step freezing demonstrates that slow cooling at a very low temperature is essential to avoid thermal shock of the sperm. In contrast, very fast cooling rate can be tolerated only within a certain temperature range. For grey mullet, black porgy and grouper, the two-step freezing, first to -100℃ and then to -196℃, are applicable, while for carp, it is 5℃/min from 2 to -7℃ and 25℃/min from -7 to -70℃, and for tilapias, it is 20℃/min directly to -35℃ and 5℃/min from -35 to -75℃.

BF5 sets in liquid nitrogen container provide different distances above the liquid nitrogen surface, and thus, milt mixture samples in vial are frozen at different freezing rates. On the dry ice a series of tiny holes were formed by a heated iron bar. Large or small-sized drops of milt mixture were applied to these holes. As a result, freezing rates could not be determined. Programmable freezer was used, because these programs help to determine the most favorable freezing protocol. Nevertheless, the most acceptable and simplest device is a common styrofoam box fitted with a polyethylene net 2 cm above the liquid nitrogen. The sample straws are placed on the net when the vapor in the closed box reached a temperature range of -90 to -100℃. This device worked well for the simple freezing of sperms of grey mullet, black porgy and grouper. The device can easily be adopted by students and farmers who usually do not have access to expensive facilities or equipment.

Hard clam sperm exposed at noncontrolled cooling rate with milt-CPA mixture at -90℃ or -80℃ for 10 min followed by -196℃ for 30 min resulted in the fertility ranges of 74.76 to 92.15% and 52.90 to 75.49% respectively. Small abalone sperm were preserved successfully after being cooled at the similar noncontrolled rate (Fig. 2). The fertility obtained from the 20-day frozen small abalone sperm was 94.80%, and for the 365-day frozen sperm it was 89.10%.

For black porgy sperm, Chao et al.[15] submerged the straws containing milt-CPA mixture in isopropanol at -10℃ and then frozen them at a rate of 2℃/min until -80℃, or held the sperm in liquid nitrogen vapour (-90 to -100℃) for 10 to 20 minutes. The 50 to 90% of the post-thawed sperm were motile and 91.50% showed fertility after 342 days. For the cichlid sperm, Chao et al.[24] cooled it rapidly to -35 ℃ and then at 5℃/min to -75℃, and stored at -196℃. The fertilization rate of the cryopreserved sperm were satisfactory. It appears that cooling rate in the freezing phase has a very wide optimal range in the cryopreservation.

IV. Cryogenical storage at -196℃

If storage temperature is maintained by refilling liquid nitrogen tank as often as needed, sperm preserved at cryotemperature of -196℃ may have an almost indefinite storage life. Many factors which affect post-thaw survival may interact one another and, therefore, it is difficult to avoid some reduction in fertilizing ability of the cryopreserved sperm.

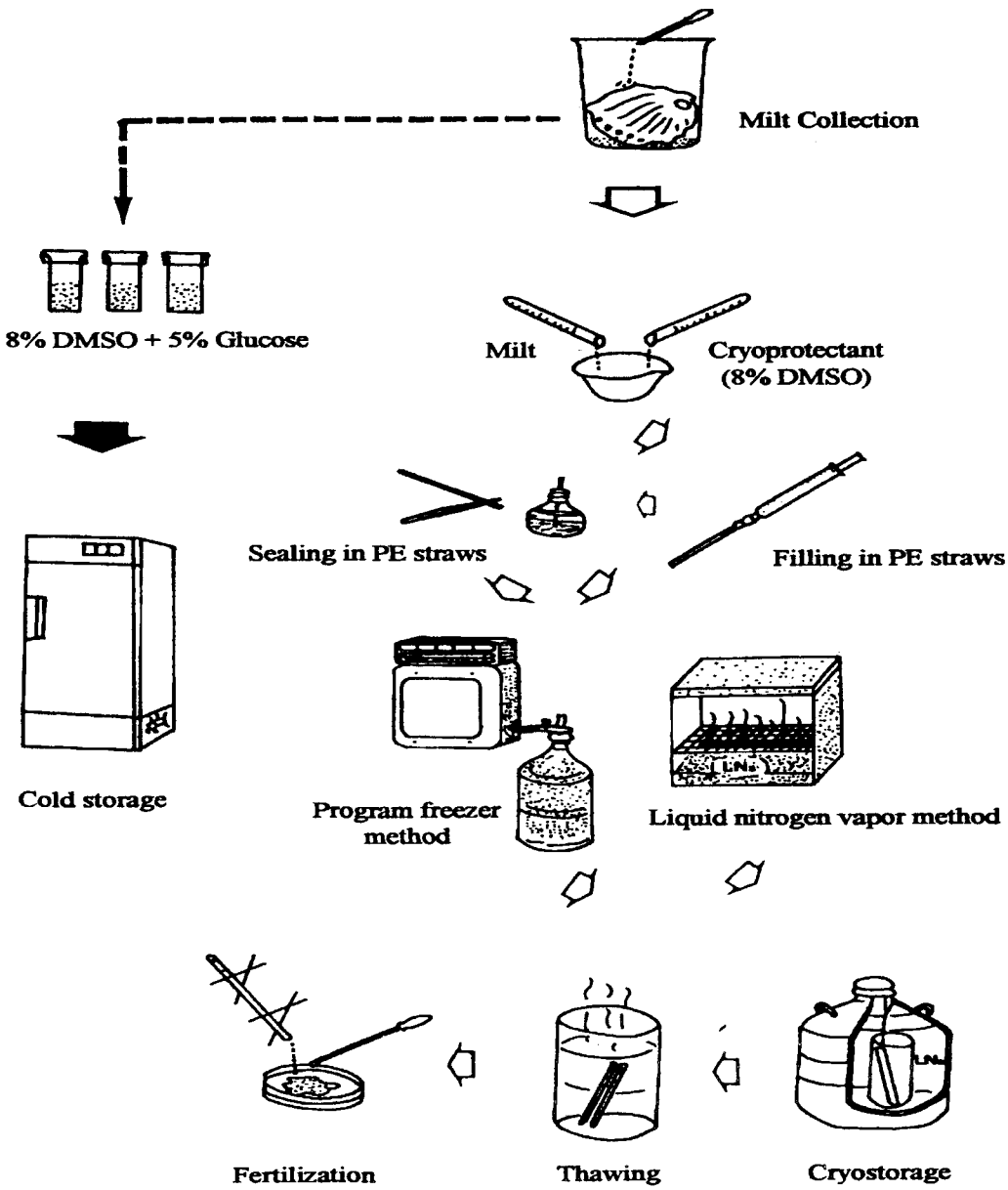

Fig. 2. A schcematic diagram of cryopreservation protocols of sperm of the small abalone *Haliotis diversicolor*.

V. Thawing of frozen sperm for fertilization

Preserved sperm is warmed from cryogenic temperature to the water temperature of the day of fertilization. Since most of the cryopreserved sperms are frozen by the PE straw method but not by the pellet method, the thawing solution to mix cryopreserved pellet is not necessary. Therefore, the room-temperature water of sufficient volume to offer the preferred highest warming rate may be used.

At the post-thaw phase, sperm that survives the cryopreservation is ready for artificial fertilization. The following procedures should be used:

(a) Estimation of sperm motility as a guide to determine their fertilization success. In our laboratory, the conventional microscopy reading and the modern motility analyzer method are compared for the estimation of the motility.

(b) Shortening the time duration between thawing and fertilization. Not only sperm strength but also duration of its motility reduce markedly in most of the experimental species with very few exceptions of which malabaricus grouper is one. Therefore, fertilization should be made immediately after thawing.

(c) Dilution ratio. Artificial fertilization using either cryopreserved sperm or fresh sperm is a function of spermatozoa concentrations. Since not all spermatozoa survive or maintain their fertility after cryopreservation, the density of frozen-thawed sperm have to be higher than that of fresh one to maintain the ideal fertility result. For *Tilapia niloticus,* 0.3 or 0.4ml milt cryopreserved at a formula of milt : milk : methanol : water = 50 : 15 : 10 : 25 are able to fertilize fresh eggs of 1g with the fertilization rate over 90%. For malabaricus grouper, the sperm quantity ranging from 0.01 and 0.08ml is sufficient to fertilize fresh eggs of 1g with significant fertility rates of 80.00% or higher, as compared to the control group of 92.00%.

In several cases of our studies, cryopreserved and fresh milt were equally effective or decreased only slightly the fertilization and hatching rates. Milt of grey mullet preserved for 351 days had the fertilization and hatching rates of 38.20 and 92.00%, respectively, while the fresh milt was 35.80 and 93.50%. Their growth rates did not differ significantly [17]. Fertilization of grouper eggs with the milt cryopreserved for 1, 17, and 291 days respectively resulted in the maximum fertilization rates of 96.72, 85.00 and 79.68%, while the fresh milt was used for fertilization of the same female eggs yileded 91.76, 80.95, and 91.92%[7]. Another evidence for the decrease in fertilizing ability of cryopreserved sperm with time was found for black porgy sperm, which showed the fertilization rates of 99.00, 93.20, 91.90 and 91.50%, respectively, after being cryopreserved for 1, 7, 7 and 342 days.

Hwang and Chen[26] indicated that when 7.5% DMSO was used, the fertilization rate of frozen-thawed oyster *Crassostrea gigas* sperm was 78.90%, while the control was 40.07%. All cryopreserved sperm of small abalone for one and two years in liquid nitrogen gave the satisfactory fertilization rates from 86.00 - 95.00%[27].

Acknowledgement

Thanks are due for assistants who worked with me on the following projects NSC 85-2321-B-056-001-A 15 and NSC 86-2311-B-056-001-B24.

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綜 論

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Journal of TFRI TAIWAN 1993 Fisheries Research

魚貝類精子冷凍保存

摘 要

有關水產生物配子超低溫保存的各種研發項目當中，不論是魚類或貝類，精子之保存其歷史均遠比卵胚胎保存者爲久且效果卓著。本文首就生物樣品之冷凍保存原理方面之降溫、解凍、超低溫儲存，以及冷凍傷害方面之酸鹼度變異、冷刺激、冰晶形成、細胞穿透性、抗凍劑毒性，分別作系統化的條列與敘述。其次，討論並評比迄今在臺灣十餘種魚貝類精液冷凍保存全面成功之主要因素，舉凡（1）取樣和評估品質，（2）供試細胞和抗凍劑之平衡，（3）精液採定速和非定速降溫，（4）超低溫保存於 -196℃，以及（5）解凍樣品、回溫、人工受精等，均提供一詳盡之回顧與綜述。

關鍵詞： 魚貝類，精液，冷凍保存

趙乃賢 (1996) 魚貝類精子冷凍保存. 水產研究, **4**(2): 157-170.