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Cryopreservation of Crustacean Gametes and Embryos

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The paper highlights the methodology that has been followed in our laboratory for freezing crab spermatozoa and the early stage larvae of the penaeid prawn *Penaeus indicus*. Gametes and embryos survive freezing to sub-zero temperatures only in the presence of a cryoprotectant. However, several cryoprotectants are toxic to the living cell and hence the ability of sperm and embryos to withstand the toxicity was evaluated. Using a programmable freezer, Kryo 10 (Planer Biomed., UK), we subjected the gametes and embryos in suitable extender media to different cooling rates using vapourised liquid nitrogen. The survival of the male gametes after thawing was tested by inducing aerosome reaction using calcium ionophore A23187. The embryos (morula) and the larvae (nauplius VI stage) were tested for survival after freezing and thawing by direct observation of hatching or motility. Using the slow cooling protocol, a survival of 82% in nauplii frozen to −30°C and 63% at −40°C was achieved. The importance of cryogenic storage of penaeid prawn embryos in alleviating the seed stocking problem in aquaculture is discussed.

Key Words : Cryopreservation, Crustacean gametes, Gametes, Embryos, Cryogenic storage

Introduction

Recent years have experienced a great deal of impetus to evolve means and methods to extend the already well established techniques of cryopreservation of gametes and embryos of mammals to those of aquatic organisms. This review summarizes the recent advances in the cryopreservation of spermatophores, spermatozoa and embryos of the cultivable crustacean species.

Materials and Methods

Cryopreservation of Spermatophores and Artificial Insemination—The first successful preservation of spermatophores was achieved in the freshwater shrimp *Macrobrachium rosenbergii* by Chow (1982) in Ringer solution for a period of 4 days maintained at 2°C. These spermatophores after attachment to the female sternum released viable sperm at the time of ovulation. This method, when extended to longer periods of storage, however resulted in the degeneration of the protective and adhesive matrices of the spermatophores. Subsequently, Chow et al. (1985) improved this technique by using 10% glycerol as a cryoprotective agent and storing the spermatophores in liquid nitrogen at −196°C up to 30 days. Since the thawed spermatophores lose the adhesiveness, these workers fastened

the cryopreserved spermatophores to the female sternum using α-cyanoacrylate as the adhesive.

The long term storage of lobster spermatophores for artificial insemination has been developed by Ishida et al. (1986). The electrically extruded *Homarus* spermatophores were transferred to a plastic test tube containing paraffin oil and stored at 4-7°C. The sperm stored up to 289 days exhibited morphological integrity as well as underwent acrosome reaction with divalent cation ionophore A23187. Long term storage of spermatophores using this method has however resulted in the degeneration as well as bacterial growth in the sperm mass. Recently, Jeyalectumie and Subramoniam (1989) have successfully frozen the spermatophores and the seminal plasma of the edible crab *Scylla serrata* at three temperatures (−4°C, −79°C and −196°C). Biochemical alterations of proteins, carbohydrates and lipids as well as the isozyme lactate dehydrogenase were also measured at these three temperatures. Whereas the metabolic activity was retained at −4°C, no such activity was recorded at −79°C and −196°C. However, the sperm viability was retained at the latter two temperatures. These authors used the dye exclusion technique (Eosin-Nigrosin) for testing the viability of the spermatozoa within the spermatophores, as the fertilizing capacity of the spermatozoa could not be tested by challenging with freshly laid eggs for want of freshly ovulated female crabs. In this respect, it may be mentioned that Anchordoguy et al. (1988) have used the acrosome reaction in the shrimp *Sicyona ingentis*, as a test to study the fertilizability of cryopreserved spermatozoa.

Although the above mentioned attempts in the cryopreservation of spermatophores and spermatozoa need perfection, they are indicative of the possibility that crustacean spermatozoa/spermatophores banking could be achieved for artificial insemination as well as for easy transportation. We have now developed techniques to release the spermatozoa from the spermatophores of *Scylla serrata* for cryopreservation, both by enzymatic digestion and mechanical means. The liberated spermatozoa were suspended in an artificial extender medium devoid of calcium. The programmed cooling of the spermatozoa has been achieved using a programmable freezer Kryo 10/1.7, Planer Biomed, UK. The importance of using suitable cryoprotectants has been studied by screening several cryoprotectants such as glycerol, ethylene glycol, methanol and dimethyl sulphoxide (DMSO).

Results

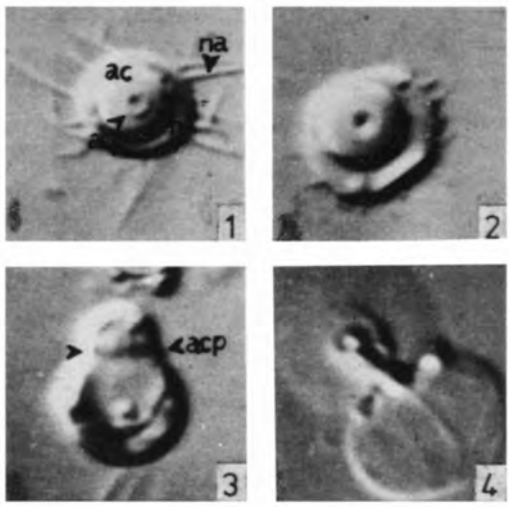
Spermatozoa were frozen in the programmable freezer at rates of −5 to −15°C/min from room temperature to various subzero temperatures. Ice nucleation was initiated by manual seeding at −7°C to ensure phased crystallization. Samples were held at −30°C for an hour and thawed to room temperature to assess freezing damage/survival. Thawing was rapid and carried out by immersing the sample holders in warm water bath maintained at 60°C, for 20-30 secs (table 2). The sperm thus frozen, underwent aerosome reaction on . treatment with ionophore A23187 (figures 1-4).

Table 1 *Tolerance to cryoprotectant at room temperature*

Equilibration at 23°C (Time in min)	% Survival in DMSO		
	0.5M	1M	1.5M
5	92±6.78	88.1±2.11	89.2±2.03
10	90.2±1.54	85.1±3.3	84 ±4.69
15	86.3±3.03	80.1±4.57	80.6±4.7

No. of observations = 10

(Bhavanishankar & Subramoniam - Unpublished data)



Figures 1-4 Acrosome reaction in *S. serrata*; **1**, Intact sperm, **2**, Retraction of nuclear arms and increase in nuclear mass, **3**, Onset of eversion of subacrosomal material through apical cap, **4**, Everted sperm (acrosome reacted) (Scale 1.2 cm = 4 μ)
n - nucleus, *ac* - acrosome, *acp* - apical cap, *na* - nuclear arms

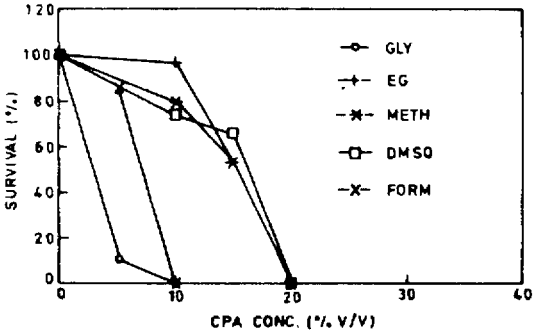


Figure 5 CPA toxicity response in penaeid prawn morulae *CPA* Cryoprotective Agent; *Gly* Glycerol; *EG* Ethylene Glycol; *METTH* Methanoll; *DMSO* Dimethyl Sulphoxide; *FORM* Formamide (From Newton & Subramoniam - Unpublished data)

Cryopreservative of embryos/larvae of penaeid prawns

As the freezing injuries to the embryos can be alleviated only by the addition of a cryoprotectant, a number of cryoprotectants were tested for their toxicity on two stages of embryonic development (i) morulae and (ii) nauplii of the penaeid prawn *P. indicus*.

Table 2 *Post-thaw survival of cryopreserved spermatozoa*

Conc. of DMSO	Freezing rate (°C/min)	Survival (%)
0.5M	−5	0
1M	−5	42.24±5.04
	−10	13.06±2.6
	−15	2.1±0.12
1.5M	−5	47.33±1.65
	−10	20.62±3.82
	−15	4.09±0.8

No. of observations = 5
(*S Bhavanishankar & T Subramoniam—Unpublished data*)

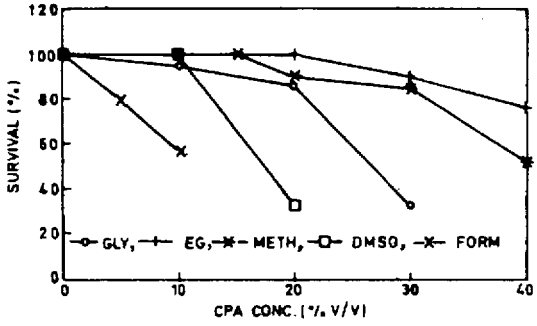


Figure 6 CPA toxicity response in iv stage nauplii; *CPA* Cryoprotective Agent; *GLY* Glycerol; *EG* Ethylene Glycol; *METH* Methanol; *DMSO* Dimethyl Sulphoxide; *FORM* Formamide (after Newton & Subramoniam - Unpublished data)

The freezing protocol employed was a 3-ramp cooling programme with a start temperature of 15°C, an overall cooling rate of −1.5°C/min and manual seeding at −6°C using a pair of tongs dipped in liquid nitrogen. On reaching final temperatures of −30°C and −40°C the frozen samples were removed from the freezing chamber of the Kryo 10 and thawed rapidly at a thawing rate >300°C/min. The toxicity response of the

morulae and nauplii were more or less similar (figures 5 and 6), except for glycerol, which was lethal to the morulae but tolerated by the nauplii at concentrations up to 30% v/v. In general, the nauplii were more tolerant to all cryoprotectants as compared with morulae. Among the two stages tested for slow cooling, success was achieved only with nauplius. On the other hand, the morulae could not survive any slow cooling programme beyond −12°C. Observation of appendage movement and subsequent active swimming movements were used as indices to evaluate survival of the frozen-thawed nauplii. A percentage survival of 82% was achieved in nauplii frozen to −30°C and 63% at −40°C.

Discussion

Techniques of cryopreservation as applied to aquatic organisms have direct application to aquaculture and conservation of threatened species as well as genetically modified or improved eggs and embryos. Although several successful attempts have been made to cryopreserve the sperm of finfishes, this has met with only limited success in the preservation of eggs and embryos. In a similar way, cryopreservation of crustacean gametes and embryos has not been achieved before, although it has tremendous application potentials to the improvement of cultivable species as well as long term storage of embryos/larvae to ameliorate the problem of seed scarcity faced by the aquaculture industry during the lean season.

Decapod crustacean sperm assume greater significance in respect of cryogenic storage. They have a natural ability to be stored in the female, pending egg release and fertilization during spawning (see Subramoniam 1991, 1993 for reviews). The crustacean spermatozoa have certain peculiar features such as a non-flagellate condition and an unusually large acrosome. Early workers who attempted to preserve the spermatozoa which are contained in the spermatophores have met with failure due to several factors such as breaking up of acrosome and the loss of spikes during prolonged storage (Ishida et al. 1986). Furthermore, the nonmotile condition of the crustacean spermatozoa also limits the viability assessment of the cryopreserved spermatozoa. On the other hand, the occurrence of a large acrosome could facilitate the easy induction of acrosome reaction by the divalent cation ionophore A23187, as has been demonstrated by Anchordoguy et al. (1988). In *Scylla serrata*, the acrosome reaction of the spermatozoa released from the spermatophores could be easily induced using such ionophores. Understandably, artificial induction of acrosome reaction can be used to test the fertilizability of the cryopreserved spermatozoa. In recent years, several attempts have been made to cross different species of crustaceans in order to obtain hybrids for improving the species for cultivation (table 3). In all these cases artificial insemination has been acheived both intraspecifically and interspecifically by means of spermatophore transfer. This requires a standardized technique for cryopreservation of spermatophores in order to transport them from far away places as well as to store them before use. This will also result in the establishment of a spermatophore or sperm bank for economically important crustacean species.

To date, there is only one published report on the successful cryopreservation of crustacean embryos (Subramoniam & Newton 1993). This study has particularly revealed the intricacies as well as the problems facing the cryopreservation of crustacean embryos. Notably, the enormous amount of yolk accumulated during oogenesis impedes successful cryopreservation in several oviparous aquatic organisms. We have found that the penaeid prawn embryos are relatively easier to cryopreserve by virtue

Table 3 *Crustacean species in which artificial isemination has been achieved*

No.	Species	Thelycum	Insemination	References
	***Penaeid* species**			
1	*P. setiferus* × *P. schmitti*	Open	Interspecific	Bray et al. 1990
2	*P. setiferus* × *P. stylirostris*	Open	Interspecific	Lawrence et al. 1984
3	*Penaeus* sp.	Open	Intraspecific	Persyn 1977
4	*Penaeus* sp.	Open	Intraspecific	Aquacop 1983
5	*P. setiferus*	Open	Intraspecific	Bray et al 1982 Bray & Lawrence 1984
6	*P. japonicus*	Closed	Intraspecific	Laubier-Bonichon and Ponticelli 1981 Lumare 1981 Ponticelli 1981
7	*P. monodon*	Closed	Intraspecific	Muthu & Laxminarayanan 1984
8	*P. vannamei*	Closed	Intraspecific	Gogueheim et al. 1987
9	*P. pencillatus*	Closed	Intraspecific	Lin & Hanyu 1991
	***Macrobrachium* species**			
1	*Macrobrachium asperulum* × *M.shokitai*	Open	Interspecific (Sterile)	Shokita 1978
2	*Palaemonetes pugid* × *P. vulgaris*	Open	Interspecific	Sandifer & Lynn 1980; Berg et al. 1986
3	*M. nipponense* × *M. formosense*	Open	Interspecific	Uno & Fugita 1972
4	*M. rasenbergii* × *M. acanthurus*	Open	Interspecific	Sandifer et al. 1977
5	*M. acanhurus* × *M. carcinus*	Open	Interspecific	Dobkin et al. 1974
6	*M. rosenbergii*	Open	Intraspecific	Sandifer & Smith 1979; Sandifer & Lynn 1980
7	*M. acanthus*	Open	Intraspecific	Sandifer et al. 1977
	Lobsters			
1	*Homarus americanus*	Closed	Intraspecific	Talbot et al. 1986 Aiken & Waddy 1980
2	*H. americanus* × *H. gammarus*	Closed	Interspecific	Hedgecock et al. 1977 Talbot et al. 1983

(From Subramoniam 1993)

of their meagre yolk content. Our unpublished results also reveal that the later stage embryos as well as the nauplius larvae are more resistant to cryoprotectant treatment than the early embryonic stages, as there is complete utilization of yolk during embryogenesis.

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