FISH GENETICS AND BIODIVERSITY CONSERVATION

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Muzaffarnagar-251 001.

1998

NATURE CONSERVATORS MUZAFFARNAGAR-251 001 (INDIA)

Fish Gen. Biodiversity Conserv.	Natcon Pub - 05	243--251	1998

Cryopreservation of spermatophores of the marine shrimp *Penaeus indicus* H. Milne Edwards

A. D. DIWAN & S. JOSEPH

Central Institute of Fisheries Education
(Deemed University) ICAR, J.P. Road, Versova, Bombay- 400 061 (India)

Abstract

Attempts on cryopreservation of spermatozoa have been made in penaeid shrimp *Penaeus indicus*. It was observed that viable spermatozoa could be preserved successfully for a period of 60 days at -35°C and -196°C temperature. Among several cryoprotectants used a combination of DMSO (5%) + glycerol (5%) and DMSO (5%) + trehalose (0.25 M) showed best viability rate (75 to 80%) in freeze thawed spermatoza. The activational changes in spermatozoa during acrosome reaction after induction with an egg water have also been mentioned. The importance of cryogenic storage of spermatozoa has been discussed.

Key words: *Penaeus indicus*, Spermatozoa, Freezing, Viability.

Introduction

Successful cryopreservation of fish gametes is well established for sperm cells from many species (Muir and Roberts, 1993) but similar attempts on invertebrate sperm particularly that of crustaceans are very limited. Chow (1982) for the first time reported the successful preservation of spermatophores of the freshwater shrimp *Macrobrachium rosenbergii*. Ishida *et al.*, (1986) later developed a technique for long term storage of lobster (*Homarus*) spermatophores. Spermatozoa of the penaeoid prawn *Sicyonia ingentis* have bee successfully preserved for a period of 2 months in liquid nitrogen by Anchordoguy *et al.* (1988). Jayalectumie and Subramoniam (1989) and Joshi and Diwan (1992) have developed a method to cryopreserve the viable spermatophores in case of mud crab *Scylla serrata* and shrimp *Macrobrachium idella* respectively. Recently, Subramoniam (1993 & 1994) has reviewed cryopreservation of gametes and embryos of few cultivable crustacean species. Inspite of the fact that among invertebrates, decapod crustaceans are the most economicaly important group of animals, very little attention has been paid on freezing and preservation of gametes.

Materials and Methods

Adult specimens of *P. indicus* (males) ranging in size 100 to 110 mm in length were collected from backwaters of Cochin and brought to the laboratory in live condition. Matured males were distinguished by the presence of united petasma and swollen white colour

at the coxae of the last pair of the pereiopods. Such animals were kept separately in 500 litres cicrular tanks containing aerated seawater (S = 30%o) and provided with a biological filter. The spermatophores required for acrosome reaction and cryopreservation studies were acquired from such males by using a modified electro-ejaculation method of Sandifer and Lynn (1980). The electro-ejaculator consisted of an electric transformer to reduce voltage, a rheostat, a volt meter and two electrodes. For acquisition of spermatophores the shrimp was held ventral side up in a plastic tray with the delivery tubes of the gill irrigator placed in each branchial cavity of the animal and a continuous flow of water bathed each set of gills. The electrodes were placed near the gonopores at the base of the 5th pereiopods and a stimulus of 6 to 12 V was applied gradually for 1 to 2 seconds. The stimulus helped in expelling single spermatophore from each gonopore. The expelled spermatophore was removed with wooden needle and used for further studies.

The viability of spermatozoa was determined by acrosomal reaction inducted with an egg water which was extracted according to the method described by Griffin *et al.* (1987). Extruded spermatophores were individually and gently homogenized to free the sprem cells using a glass tissue grinder in filtered seawater (S = 30%o). Tissue fragments were separated from the sperm supernatant by hand centrifugation. Assays for acrosome reaction were conducted in culture tubes of 3 ml capacity. Prior to addition of spern, 900 l of egg water was added to each assay tube. Then immediately the supernatant was removed by a pipette and 100 ml of sperm cells were pipetted into the egg water and mixed throughly. After 5 minutes of incubation 100 ml of the sample was removed to another tube and fixed with a drop of 70% ethanol in seawater (as control). The remainder of the sample was allowed to incubate for another 55 minutes. After 1 h incubation, sperm were examined to determine reacted sperm cells which had undergone acrosomal exocytosis as described by Clark *et al.* (1981). The reacted sperm cells were counted using haemocytometre and observing under phase-contrast microscope. Each time more than 100 sperm cells were counted and average of three such counts were taken into consideration to represent reacted spermatozoa in each sample.

The cryoprotectants tested and used in the present study were dimethyl sulfoxide (DMSD 5%) and Glycerol (5%) both prepared in standard seawater (S = 35%0), combination of DMSO (5%) and glycerol (5%) (1:1), DMSO (5%) and trehalose (0.25 M) (1:1) and trehalose (0.25 M) alone. For cryopreservation studies multiple sets of cryovial (5 ml capacity) made of polycarbonate were taken in triplicate for each of the cryoprotectants to be tested. Sperm cells isolated from spermatophores by the method described above were diluted in a extender solution (Standard Seawater) to a concentration of 10^6 to 10^7 sperm/ml and later incubated in an equal volume of cryoprotectant for 5 minutes at room temperature before initiation of the cooling process. Three different temperatures tested were 0°C, -35°C and -196°C. Freezing chamber of refrigerator was used for 0°C and for -35°C a programmable cryostat was used and for -196°C temperature, the samples were stored in liquid nitrogen (LN_2). Sperm samples incubated with cryoprotectants were frozen in the cryovials at the precise cooling rates using programmable cryostat. The samples were cooled from room temperature to -35°C at a rate of -1°C per minute. Once the temperature reached -35°C, the

samples were then exposed to the vapours of LN_2 for 5 minutes before plunging the same into LN_2 permanently. Percentage of viable sperm was determined at three different temperatures at periodical intervals of 7, 15, 30 and 60 days respectively. To determine the viability of cryopreserved sperm, the samples were thawed at room temperature -20°C. When the sperm suspensions melted completely, the samples were diluted with 900 l of standard seawater and centrifuged in a refrigerated centrifunge machine for 3 minutes. The supernatant was removed and the sperm pellet was resuspended in 100 l of egg water for 1 h and then the percentage of sperm that had undergone both phases (exocytosis and filament formation) of acrosome reaction was determined.

Results

The morphological features of non-motile (un-reacted) spermatozoa in case of *P. indicus* when observed under phase-contrast microscope revealed that superficially the sperm are distinguished into three regions viz. (1) a posterior main body, (2) a central cap region and (3) an anterior spike. The posterior main body is an elongate sphere housing an uncondensed nucleus followed by a central cap region which includes acrosomal vesicles. Literally though the penaeid sperm appear to be non-motile they become active just prior to fertilization. The activated or reacted sperm exhibited a total change in their morphological anatomy. It has been found that in the wild when sperm cell comes in contact with an egg at the time of spawning, it binds with the tip of the spike to eggs' viltelline envelope and in no time gets activated. The first manifestation of an activated sperm is the loss of anterior spike which immediately results in the externalization of the acrosomal vesicle contents and exocytosis of the acrosomal vesicles. With the exocytosis of the acrosomal vesicles, sperm activation is completed by forming a long acrosomal filament (Fig. 1). Similar changes occur when sperm

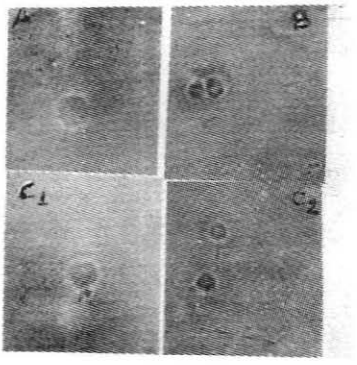

Fig. 1 : Phase micrographs depiciting the acrosome reaction of *P. indicus* sperm.
(A) An unreacted sperm.
(B) A sperm that has lost the spike and undergone exocytosis of the acrosomal vesicle (C_1 C_2) Sperm with acrosomal filament.

Table 1 : Actual counts and percentage of activated spermatozoa of *Penaeus indicus* cryopreserved at 0°C for different duration of time.

Duration of Cryopreservation	Activated spermatozoa				
	Spermatozoa with spike	Exocytosis	Filament formation	Percentage of activated spermatoza	Cryoprotectants used
	33±2	37±3	33±3	67.0	Glycerol
	51±2	60±2	51±2	68.8	DMSO
7 days	30±3	40±2	30±3	70.0	Glycerol±DMSO
	36±2	37±2	40±3	69.0	DMSO±Trehalose
	52±2	45±2	37±2	63.8	Trehalose
	-	-	-	-	Control
	37±4	36±3	34±5	65.85	Glycerol
	43±5	37±5	37±5	63.09	DMSO
	43±4	50±6	40±6	67.76	Glycerol±DMSO
15 days	34±3	42±5	34±5	68.83	DMSO±Trehalose
	41±3	30±5	30±5	60.01	Trehalose
	23±2	7±1	-	-	Control
	46±5	19±2	34±2	53.48	Glycerol
	43±5	25±5	17±1	49.86	DMSO
	55±4	30±3	48±1	58.22	Glycerol±DMSO
30 days	52±3	40±5	45±5	61.55	DMSO±Trehalose
	42±3	15±3	16±4	42.46	Trehalose
	14±2	0.5	-	-	Control

Each value is mean of three determinations.

Table 2: Actual counts and percentage of activated spermatozoa of *Penaeus indicus* cryopreserved at-35°C for different duration of time.

Duration of Cryopreservation	Activated spermatozoa				
	Spermatozoa with spike	Exocytosis	Filament formation	Percentage of activated spermatoza	Cryoprotectants used
	16±5	25±3	15±5	70.75	Glycerol
	25±5	28±4	33±4	70.54	DMSO
7 days	15±5	29±5	28±5	79.38	Glycerol±DMSO
	16±4	32±6	33±6	79.87	DMSO±Trehalose
	21±6	26±4	21±24	69.00	Trehalose
	22±5	4±1	-	-	Control
15 days	36±6	39±5	35±5	67.20	Glycerol
	27±5	37±4	35±5	65.79	DMSO
	29±5	55±4	36±5	75.94	Glycerol±DMSO
	31±3	58±5	50±4	77.80	DMSO±Trehalose
	40±6	32±4	30±5	62.87	Trehalose
	33±5	10±3	—	—	Control
30 days	37±6	33±5	38±5	64.90	Glycerol
	54±1	33±4	48±5	59.39	DMSO
	40±6	41±5	57±5	70.57	Glycerol±DMSO
	33±5	28±5	54±6	74.63	DMSO±Trehalose
	36±5	16±5	31±4	57.89	Trehalose
	17±2	9±2	—	—	Control
60 days	42±3	22±4	33±4	56.70	Glycerol
	60±5	28±3	43±4	54.19	DMSO
	60±4	53±4	51±5	63.41	Glycerol±DMSO
	36±5	33±5	48±3	69.23	DMSO±Trehalose
	77±6	36±4	41±3	50.00	Trehalose
	36±3	9±2	-	-	Control

Each value is mean of three determinations.

Table 3 : Actual counts and percentage of activated spermatozoa of *Penaeus indicus* cryopreserved at -196°C for different duration of time.

Duration of Cryopreservation	Activated spermatozoa				
	Spermatozoa with spike	Exocytosis	Filament formation	Percentage of activated spermatoza	Cryoprotectants used
	47±4	82±5	56±5	74.22	Glycerol
	50±2	64±4	53±4	70.07	DMSO
7 days	26±3	56±5	39±2	78.57	DMSO±Glycerol
	25±3	59±5	56±5	81.92	DMSO±Trehalose
	48±1	55±5	49±3	68.46	Trehalose
	67±2	10±3	-	-	Control
15 days	49±3	64±3	54±2	70.7	Glycerol
	48±1	59±2	47±3	68.90	DMSO
	35±2	71±3	55±3	78.19	Glycerol±DMSO
	26±2	57±3	55±4	80.39	DMSO±Trehalose
	39±4	35±4	29±3	62.72	Trehalose
	28±2	9±2	-	-	Control
30 days	42±4	47±3	40±3	67.47	Glycerol
	42±3	47±4	42±3	67.99	DMSO
	30±2	57±5	40±3	75.52	Glycerol±DMSO
	27±4	55±4	40±3	78.10	DMSO±Trehalose
	31±3	22±3	24±3	59.89	Trehalose
	17±1	7±2	-	-	Control
60 days	50±5	35±5	38±4	58.70	Glycerol
	46±4	24±4	38±3	56.67	DMSO
	42±4	38±3	49±4	67.16	Glycerol±DMSO
	37±4	27±4	58±5	69.97	DMSO±Trehalose
	53±5	27±3	35±3	53.73	Trehalose
	42±5	15±3	-	-	Control

Each value is mean of three determinations.

in-vitro are inducted with an egg water.

Among the five cryoprotectants tested, the performance of DMSO combined with glycerol and DMSO with trehalose was found to be the best, as in these solution viability of freeze thawed spermatozoa was found to be the highest (60 to 80%) even in the extended period of 60 days of cryopreservation at -35°C and -196°C (Table 2, 3). DMSO, glycerol and trehalose when used independently showed low percentage of viability of freeze thawed spermatozoa cryopreserved at all the three temperatures viz. 0°C, -35°C and -196°C (Table 1-3).

Discussion

In recent times due to awareness of cryogenic preservation of fertile gametes to enhance animal production in controlled condition, extensive studies are being made on sperm-egg characteristics of several commercially important species. In decapod crustaceans, particularly in marine shrimps, such studies are meagre. In crustaceans, the sperm are reported to be always non-motile and non-flagellabted (Felgenhauer and Abele, 1991). Several attempts have beem made to reveal the structural details of the crustacean spermatozoa through SEM and TEM studies. Lynn and Clark (1983) have done SEM studies on sperm egg interaction in freshwater prawn *M. rosenbergii* whereas in penaeid prawn *Sicyonia ingentis,* Clark *et al.* (1981) made indepth investigation on sperm-egg activational changes through ultrastructural means. Two phases of acrosomal reaction i.e., exocytosis of the acrosomal vesicle and generation of the acrosomal filament as noticed in the inducted spermatozoa of *P. indicus* of the present study have been identically reported in other penaeid like *S. ingentis* (Clark *et al.* 1981, 84 and Griffin *et al.* 1988). For induction of spermatozoa, among different compounds like bromo-calcium ionophore, valinomycin, nigricin and egg-water, the egg-water has been reported to be the best inducting agent for acrosome reaction (Griffin *et al.* 1987). Later, *In-vitro* induction of acrosomal filament in the natantian sperm with an egg water has been achieved by many investigators (Griffin *et al.* 1988; Griffin and Clark, 1990; Clark and Griffin 1993; Pratoomchat *et al.* 1993). Therefore, the sperm viability test in the present investigation was done only with egg-water. The morphologically identifiable structural changes of the sperm during acrosome reaction infact made the viability studies easy which is the basic information required for further manipulaion of the sperm.

There are very few reports on cryopreservation of crustacean sperm. The temperature at which the sperm samples are stored as well as cryoprotectants used for dilution of sperm have definite and significant role on the achievement of viability of cryopreserved sperm in *P. indicus* in the present study. From the results it is found that - 196°C is the best temperataure for storage of intact viable sperm for longer periods than other two temperature i.e. -35°C and 0°C. Among the cryoprotectants, mixture of DMSO and glycerol and DMSO and trehalose have been found to be the best preservative media. Since glycerol was first reported as effective in protecting sperm from freeze-thaw damage by Rostand (1946), this has been widely used as a cryoprotectant in cryopreservation studies. Using glycerol as a

cryoprotectant, Chow *et al.* (1985) could preserve spermatozoa of shrimp successfully at -196°C temperature but the same in DMSO did not show encouraging results. Anchordoguy *et al.* (1988) reported that the sperm of *S. ingentis* stored in glycerol at -195°C for 1 month showed no decrease in viability upon thawing. In *Scylla serrata,* the viability of the sperm in 30 days of storage was fairly high (95%) in samples stored in glycerol and DMSO + trehalose at -196°C (Jeyalectumie and Subramoniam, 1989). In *S. ingentis* Anchordoguy *et al.* (1988) tested different cooling rates and it was reported that a coolng rate of 1°C/min was the best in cryopreservation studies. The same cooling rate was used in the present investigation also.

In the present study, glycerol and DMSO when used independently as cryoprotectants, percentage of viability of spermatoza was comparatively better at all temperatures tested. Only in trehalose there was almost 50% mortality. Anchordoguy *et al.* (1988) reported the high percentage of viable sperm in shrimp *S. ingentis* from the samples preserved in 5% DMSO. With regard to trehalose as a cryoprotecting media, Jeyalectumie and Subramoniam (1989) reported that it is not an efficient medium when used independently. Similar findings have been reported by Anchordoguy *et al.* (1988) while working on cryopreservation of sperm in *S. ingentis*. From the results of the present study it is concluded that viable spermatophores of penaeid praws can be stored for longer duration through cryopreservation techniques. As the mariculture industry is developing rapidly, many experimental studies are required to evolve suitable technologies on these lines and develop patents.

Acknowledgements

Authors wish to express their sincere thanks to Dr. M. Devaraj, Director, Central Marine Fisheries Research Institute, Cochin for providing facilities and encouragement.

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