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

A.D. DIWAN[1] AND SHOJI JOSEPH

Central Marine Fisheries Research Institute, Cochin - 682 014, India

ABSTRACT

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

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 those of crustaceans are very limited. Chow (1982), for the first time, reported the successful preservation of spermatophores of freshwater shrimp, *Macrobrachium rosenbergii.* Ishidae^aZ. (1986) later developed a technique for long-term storage of lobster *(P. homarus)* spermatophores. Spermatozo of the penaeid prawn, *Sicyonia ingentis,* have been successfully preserved for a period of two months in liquid nitrogen by Anchordoguy *et al.* (1988). Jeyalectumie and Subramoniam (1989) and Joshi and Diwan (1992) have developed a method to cryopreserve the viable spermatophores of mud carb, *Scylla serrata* and shrimp, *Macrobrachium idella,* respectively. Recently Subramoniam (1993, 1994) has reviewed cryopreservation of gametes and embryos of a few cultivable crustacean species. In spite of the fact that among invertebrates decapod crustaceans are the most economically 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) 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 pereiopods. Such animals were kept separately in 500 1 circular tanks containing aerated seawater (S » 30 *%o)* and provided with a biological

Present Address: [1] Central Institute of Fisheries Education (Deemed University), Versova, Mumbai - 400 061, India.

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 viability of spermatozoa was determined by acrosomal reaction induced with an egg water which was extracted according to the method described by Griffin *et al.* (1987). Extruded spermatophores were individually and gently homogenised to free the sperm 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 ul capacity. Prior to addition of sperms, 900 ul of of egg water was added to each assay tube. Then immediately the supernatant was removed by a pipette and 100 pi of sperm cells were pipetted into the egg water and mixed thoroughly. After 5 minutes of incubation, 100 pi 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 of incubation, the sperms 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 was taken into consideration to represent reacted spermatozoa in each sample.

The cryoprotectants tested and used in the present study were dimethylsulphoxide (DMSO 5 %) and glycerol (5 %) both prepared in standard seawater (S « 35 *%o),* 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 cryovials (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, for -35°C a programmable cryostat was used and for -196°C the samples were stored in liquid nitrogen (LN_2). Sperm samples incubated with cryoprotectants were frozen in the cryovials at precise cooling rates using programmable cryostat. The samples were cooled from room temperature to -35^{C}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 viability of sperm was determined at three different temperatures at 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 pi of standard seawater and centrifuged in a refrigerated centrifuge for 3 minutes. The super-natant was removed and the sperm pellet was resuspended in 100 pi of egg water for lh and then the percentage **of sperm** that had undergone both phases (exocytosis and filament **formation) of acrosome** reaction, was **determined.**

Results

-35°C and **-196°C.** DMSO, glycerol and trehalose, when used independently, showed low percentage of viability of freeze thawed **spermatoza cryopreserved** at **all the** three temperatures ***viz.* 0°C, ~35°C** and **-196°C (Tablel-3).**

Fig, **1. Phasemicrographs of the** five **aclivational** stages of ***Penaeus indicus*** spermatozoa x 400. (A) an **unreacted** spermatozoa!! possessing an **anterior spike, (B)** a **spermatozoan that** has undergone acrosomal exocytosis and has lost the spike, (0) initiation of **acrosomal** filament **formation, (D) acrosomal** filament formation and (E) a **spormatozoan that** has completed **the** acrosome reaction by forming an acrosomal **filament.**

TABLE 1. Actual counts and percentage of activated spermatozoa of Penaeus indicus *cryopreserved at -0°C for different durations*

Duration of cryopreservation	Activated spermatozoa: Spermatozoa with spike	Exocytosis	Filament formation	Percentage of activated spermatozoa	Cryoprotectants used
At time zero	25±2	45±3	46±2	78.0	Glycerol
	16±1	53±4	54±3	88.0	DMSO
	18±3	51±3	57±3	86.0	Glycerol+DMSO
	20±2	48±2	51±2	83.0	DMSO+Trehalose
	22±3	44±2	49±2	81.0	Trehalose
	24±2	50±2	53±2	81.0	Seawater
7 days	33±2	37±3	33±2	67.0 a	Glycerol
	51±2	60±2	52±2	68.8 a	DMSO
	30±3	40±2	30±3	70.0 a	Glycerol+DMSO
	36±2	37±2	40±3	69.0 a	DMSO+Trehalose
	52±2	45±3	37±2	63.8 a	Trehalose
					Control
15 days	37±4	36±3	34±5	65.85 a	Glycerol
	43±5	37±5	37±5	63.09 a	DMSO
	43±4	50±6	40±6	67.76 a	Glycerol+DMSO
	34±3	42±5	34±5	68.83 a	DMSO+Trehalose
	41±3	30±5	30±5	60.01 a	Trehalose
	23±2	7±1			Control
30 days	46±5	19±2	34±2	53.48 a	Glycerol
	43±5	25±5	17±1	49.86 a	DMSO
	55±4	30±3	48±1	58.22 a	Glycerol+DMSO
	52±3	40±5	45±5	61.55 a	DMSO+Trehalose
	42±3	15±3	16±4	42,46 a	Trehalose
	14±2	05±0	-	-	Control

Each value is mean of three determinations ± SD. a : $P<0.001$.
The percentage of acrosome reaction on freshly collected spermatozoa is observed to be between 85-90.
Control: Standard seawater used as medium (S = 35‰).

Discussion

In crustaceans, the sperms are reported to be always non-motile and non-flagellated (Felgenhauer and Abele, 1991) Several attempts have been 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 in depth investigations 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 induced spermatozoa of P. *indicus* of the present study, have been identically reported in the other penaeid like *S. ingentis* (Clark e£ *al.,* 1981, 1984 and Griffin *et al.,* 1988). For induction of spermatozoa, among different compounds like bromocalcium ionophore, valinomycin, nigricin and egg water, the egg water has been reported to be the best inducing agent for acrosome reaction (Griffin *et al.* 1987). Later *in vitro*

TABLE 2. *Actual counts and percentage of activated spermatozoa of* Penaeus indicus *cryopreserved at -35°C for different durations*

Duration of cryopreservation	Activated spermatoza: Spermatozoa with spike	Exocytosis	Filament formation	Percentage of activated spermatozoa	Cryoprotectants used
At time zero	23±2	46±3	51±3	80.80	Glycerol
	17±2	55±3	50±2	86.00	DMSO
	15±1	52±3	51±2	87.20	Glycerol+DMSO
	21±1	49±2	50±2	82.50	DMSO+Trehalose
	22±2	43±3	49±3	80.70	Trehalose
	25±3	51±3	54±2	80.10	Seawater
7 days	16±5	25±3	15±5	70.75 n	Glycerol
	25±5	28±4	33±4	70.54 a	DMSO
	15±5	29±5	28±5	79.38 n	Glycerol+DMSO
	16±4	32±6	33±6	79.87 n	DMSO+Trehalose
	21±6	26±4	21±24	69.00 a	Trehalose
	22±5	4±1			Control
15 days	36±6	39±5	35±5	67.20 a	Glycerol
	27±5	37±4	35±4	65.79 a	DMSO
	29±5	55±6	36±5	75.94 n	Glycerol + DMSO
	31±3	58±5	50±4	77.80 n	DMSO+Trehalose
	40±6	32±4	30±5	62.87 a	Trehalose
	33±5	10±3			Control
30 days	37±6	33±5	38±5	64.90 n	Glycerol
	54±1	33±4	48±5	59.39 a	DMSO
	40±6	41±5	57±5	70.57 n	Glycerol+DMSO
	33±5	28±5	54±5	74.63 n	DMSO+Trehalose
	36±5	16±5	31±4	57.89 a	Trehalose
	17±2	9±2			Control
60 days	42±3	22±4	33±4	56.70 a	Glycerol
	60±5	28±3	43±4	54.19 a	DMSO
	60±4	53±4	51±5	63.41 a	Glycerol+DMSO
	36±5	33±5	48±3	69.23 a	DMSO+Trehalose
	77±6	36±4	41±3	50.00 a	Trehalose
	36±3	9±2	-	-	Control

Each value is mean of three determinations ± SD. a : P<0.001. n : not significant compared to time zero values. Control : Standard seawater used as medium (S " 35‰).

induction of acrosomal filment 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 the egg water. The morphologically identifiable structural changes of the sperm during acrosome reaction in fact made the viability studies easy.

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

TABLE 3. *Actual counts and percentage of activated spermatozoa of* Penaeus indicus *cryopreserved at •196°C for different durations*

Duration of cryopreservation	Activated spermatoza: Spermatozoa with spike	Exocytosis	Filament formation	Percentage of activated spermatozoa	Cryoprotectants used
At time zero	23±2	51±2	54±3	82.00	Glycerol
	18±2	56±2	55±3	86.00	DMSO
	16±0	53±2	55±3	87.00	Glycerol+DMSO
	21±4	51±3	47±2	81.80	DMSO+Trehalose
	25±2	48±2	45±3	79.30	Trehalose
	23±2	52±3	51±3	81.00	Seawater
7 days	47±4	82±5	56±5	74.22 n	Glycerol
	50±2	64±4	53±4	70.07 a	DMSO
	26±3	56±5	39±2	78.57 n	Glycerol+DMSO
	25±3	59±5	56±5	81.92 n	DMSO+Trehalose
	48±1	55±5	49±3	68.46 a	Trehalose
	67±2	10±3	-	-	Control
15 days	49±3	64±3	54±2	70.67 n	Glycerol
	48±1	59±2	47±3	68.90 a	DMSO
	35±2	71±3	55±3	78.19 n	Glycerol+DMSO
	26±2	57±3	55±4	80.39 n	DMSO+Trehalose
	39±4	35±4	29±3	62.72 a	Trehalose
	28±2	9±2	-	-	Control
30 days	42±4	47±3	40±3	67.46 a	Glycerol
	42±3	47±4	42±3	68.90 a	DMSO
	30±2	57±5	40±3	75.52 n	Glycerol+DMSO
	27±4	55±4	40±3	78.10 n	DMSO+Trehalose
	31±3	22±3	24±3	59.89 a	Trehalose
	17±1	7±2			Control
60 days	50±5	35±5	38±4	58.70 a	Glycerol
	46±4	24±4	38±3	56.67 a	DMSO
	42±4	38±3	49±4	67.16 a	Glycerol+DMSO
	37±4	27±4	58±5	69.97 a	DMSO+Trehalose
	53±5	27±3	35±3	53.73 a	Trehalose
	42±5	15±3	-	-	Control

Each value is mean of three determinations ±SD. a : P<0.001. n : not significant compared to time zero values. Control : Standard seawater used as medium (S = 35‰).

cryopreserved sperm in *P. indicus*. From the results it is found that -196°C is the best temperature for storage of intact viable sperm for longer periods than the other two temperatures 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 the 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 but the same did not show encouraging results in DMSO. 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 glycerol, and DMSO + trehalose at - 196°C (Jeyalsetumie and Subramoniam, 1989). Anchordoguy *et al.* (1988) tested different cooling rates and it was reported that a cooling rate of l°C/min. was the best in cryopreservation studies. The same cooling rate was also used in *P. indicus.*

In the present study, glycerol and DMSO when used independently as cryoprotectants, percentage of viability of spermatoza was comparatively better at all temperature 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 medium, 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 prawns can be stored, for longer duration through cryopreservation techniques.

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