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CRYOPRESERVATION OF SEMEN FROM EDIBLE ROCK LOBSTER, *PANULIRUS HOMARUS*

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Abstract : Rock lobsters, Panulirus homarus, collected from Kanyakumari coast, South India are of export quality with good demand in international market. Their catch is dwindling in recent years which prompted us to study their semen quality before and after cryopreservation at subzero temperature for preservation and semen banking. The semen separated from spermatophore was cryopreserved for a period of 25 days at -196 °C in liquid nitrogen. Phosphate buffer was used as standard diluent. In order to assess the efficiency of various cryoprotectants which are used to prevent the sperm cell damage during cryopreservation, glecerol, dimethylsulphoxide (DMSO), methanol and glucose were used in different combinations. Crustacean sperm cells lack tail which is present in all other animal groups. So it is not possible to check the viability of sperm cells from their active motility. Therefore, the survivability of sperm cells was determined through eosin-nigrosin dye exclusion method where the live sperm cells do not absorb dye. Among the different cryoprotectants used, a combination of DMSO (5%) + glycerol (10%) showed best survivability rate (90 to 61%) than the combination of DMSO (5%) + glucose (0.25m) + methanol (10%). Quantification of major organic constituents such as total protein, total free sugar and total lipid revealed more fluctuations for E4 and least for E1. There was a decline (P < 0.001 level) in all these components reflecting their utilization in the metabolic activities of spermatozoa during the cryopreservation. The significance of cryopreserving the lobster spermatozoa is discussed.

Key words: Cryopreservation, lobster spermatozoa

INTRODUCTION

Compared to eggs, the sperm cells are preferred for cryopreservation owing to their large number, ease of collection and suitability. In fin fishes motility of sperm cells is induced after the discharge of sperm into the aqueous environment or in the female genital tract. However, crustaceans produce immotile spermatozoa. As there is no expenditure of energy in spermatozoa before fertilization, their life span is considerably increased besides facilitating flourishing storage from a few hours to several days.

Though cryopreservation of gametes was first started in animal husbandry, due to the success achieved it has been extended to aquaculture as well. In latter, cryopreservation of sperm cells has been extensively studied in fin fishes. The use of cryopreserved gametes (sperm and egg) in research and development programmes of aquaculture has excellently been reviewed by Stoss [1] and Muir and Roberts [2]. However, similar attempts on invertebrate sperm, particularly those of crustaceans are quite limited. Chow [3] for the first time reported the successful spermatophore preservation of freshwater shrimp, *Macrobrachium rosenbergii*. Spermatozoa of the penaeid prawn, *Sicyonia ingentis*, have been preserved for a period of two months in liquid nitrogen by Anchordoguy et al. [4] method. Jeyalectumie and Subramoniam [5] and Joshi and Diwan [6] have

developed a method to cryopreserve the viable spermatophores of mud crab, *Scylla serrata* and shrimp, *Macrobrachium idella* respectively.

Cryopreservation of lobster sperm cells has not been studied except the pioneering work of Talbot et al. [7]. Presently, the demand for lobster is increasing all over the world. In spite of the fact that lobsters are the most economically important group of animals, very little attention has been paid to freezing and preservation of their gametes. The objectives of this work, therefore are (1) to see the survivability of cryopreserved sperm cells (2) impact of cryoprotectant on cryopreservation and biochemical components of the semen and finally (3) to identify the best combinations of cryoprotectant for the cryopreservation of lobster semen

MATERIAL AND METHODS

Adult males *of P. homarus* (270 ± 20gm) collected from Chinnamuttom and Kadiapattanam seashore of Kanyakumari coast were transported to the laboratory and cultured in fiberglass tanks containing seawater with proper aeration. The semen was collected by dissecting out the spermatophore and was suspended in extender.

Dimethyl sulfoxide (DMSO – 5%), glycerol (10%), glucose (0.25m) and methanol (10%) were the cryoprotectants used in different combinations [glycerol + glucose (E1), 5% DMSO + glucose (E2), 5% DMSO + methanol + glucose (E3) and 5% DMSO + glycerol (E4)]. Cryoprotectants were added to the extenders mixed with *P. homarus* semen suspension. Diluents were mixed with semen in the ratio 3:1 following Scott and Bayness [8] and Munkittrick and Moccia [9]. The semen sample diluted with cryoprotectants (E1, E2, E3 and E4) and extenders were filled in 0.5ml straws, gradually cooled from room temperature to -196 °C and stored in liquid nitrogen. The percentage survivability and biochemical changes were determined at intervals of 5 days upto 25 days.

The survivability of spermatozoa was determined by using dye exclusion method using eosin – nigrosin technique [10]. Unlike the uptake of eosin by dead cells, viable cells were not stained. The percent survivability of sperm cells was calculated by dividing the number of live sperm cells with total number of sperm cells multiplied by 100.

Biochemical components such as total protein [11], total free sugar [12] and total lipid [13] in the control and cryopreserved samples were estimated once in five days upto twenty five days. The effect of cryoprotectants on survivability and biochemical changes before, during and after cryopreservation were statistically analysed for their significance [14,15], their level given at $p<0.001$, $p<0.01$ and $p<0.05$.

RESULTS

Among the different cryoprotectants used for cryopreservation of *P.homarus* semen, percentage survival of spermatozoa was lowest with glycerol + glucose. Only 47.5% survival was recorded on 25th day, the decrease being statistically highly significant ($p<0.001$). Survival of freeze-thawed spermatozoa was found to be highest (90.6 - 61.4%) when stored in 5% DMSO and glycerol (E4) combination ($p<0.05$) up to 25 days of cryopreservation (-196°C). When DMSO was mixed with methanol and glucose the percent survivability of spermatozoa was 85.6% (fifth day) and 56.4% (25th day); while 83.8% (fifth day) and 50.18% (25th day) survivability was recorded when DMSO was used as cryoprotectant with glucose. Minimum survival of 74.2% on 5th day and 47.2% on 25th day was observed for glycerol and glucose combination without DMSO (table 1 and fig. 1).

The total protein and total free sugar content of cryopreserved lobster semen decreased significantly ($P < 0.001$) up to 25 days (Figs. 2 and 3). Minimum quantities of these compounds were recorded for glycerol and glucose combination followed by DMSO with glucose combination whereas they were comparatively higher for DMSO and glycerol

Table 1: Surviability (%) of *P. homarus* Spermatozoa cryopreserved (-196^{0}C) with different cryoprotectants up to 25 days. P<0.001*** p<0.01** p<0.05*

Cryoprotectant	Control	5	10	15	20	25
Glycerol (E1) + Glucose	94.4 ± 2.15	74.2 ± 0.75	71.4 ± 1.01	63.8 ± 0.75	51.4 ± 1.01	47.2 ± 1.17***
5% DMSO+glucose (E2)	94.4 ± 2.15	83.8 ± 1.11	75.4 ± 0.48	65.8 ± 0.89	60.8 ± 0.75	50.18 ±0.75***
5% DMSO + Methannol + glucose (E3)	94.4 ± 2.15	85.6 ± 1.01	76.8 ± 1.17	68 ± 1.17	60.2 ± 0.75	56.4 ± 1.10**
5% DMSO + glycerol (E4)	94.4 ± 2.15	90.6 ± 1.01	83.6 ± 1.56	74 ± 1.41	67 ± 1.41	61.4 ± 1.01*

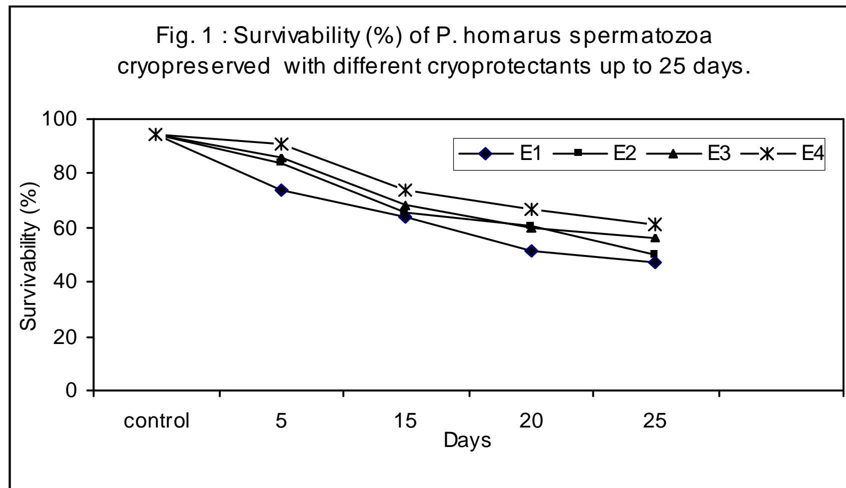


Fig. 1 : Survivability (%) of P. homarus spermatozoa cryopreserved with different cryoprotectants up to 25 days.

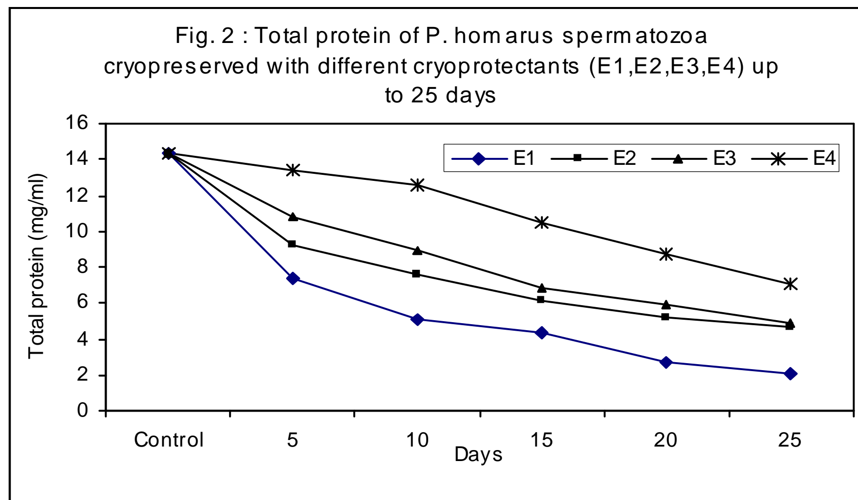


Fig. 2 : Total protein of P. homarus spermatozoa cryopreserved with different cryoprotectants (E1,E2,E3,E4) up to 25 days

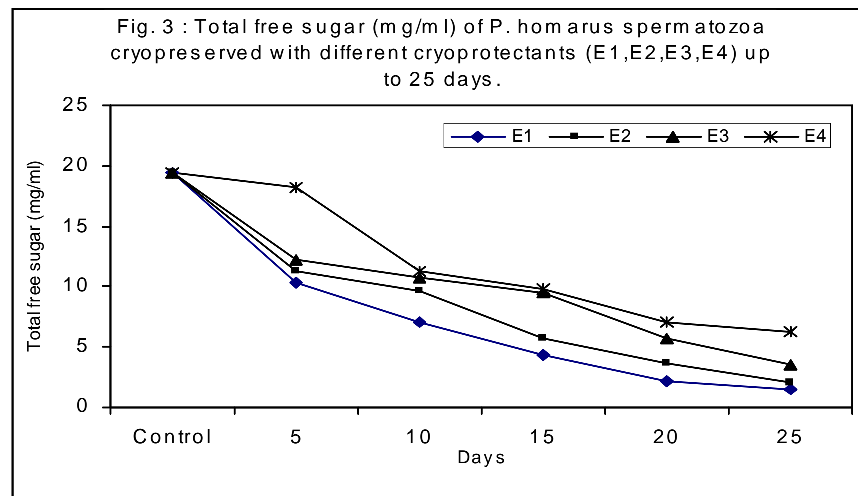
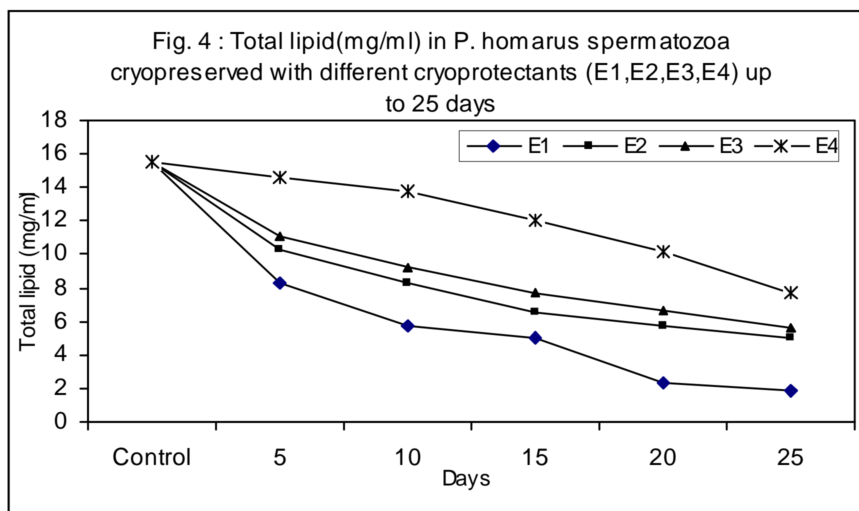


Fig. 3 : Total free sugar (mg/ml) of P. homarus spermatozoa cryopreserved with different cryoprotectants (E1,E2,E3,E4) up to 25 days.



combination (p<0.05) where the survival rate was maximum. Similarly total lipid was also found to be maximum in semen cryopreserved with DMSO and glycerol (high survival of sperm cells) as shown in figure 4.

DISCUSSION

Cryopreservation of semen, which is extensively used in animal husbandry, has been extended to fishes as well for aquaculture purpose and it has become successful to a great extent. The cryoprotectants used for the dilution of semen before cryopreservation have definite and significant role in achieving better viability of cryopreserved sperm. Like any other crustaceans, the lobster sperm cells are also non motile as they are devoid of tail [16]. Cryopreservation of crustacean semen has not been studied extensively except for the work of Chow [17] in *Macrobrachiam rosenbergii*, Anchordoguy et al. [4] in *Sicyonia ingentis*, Jeyalectumie and Subramoniam [5]in *Scylla serrata*, Diwan and Shoji [18] in *Penaeus indicus*. However, meager attention has been given towards the cryopreservation of lobster spermatozoa except for the work of Talbot [7] and her co-workers in *Homarus americanus*. Cryopreservation of lobster semen can go a long way in maintaining the biodiversity of these species.

Diwan and Shoji [18] recorded 86-61% survivability of *Penaeus indicus* spermatozoa by cryopreserving (-196ºC) in DMSO and glycerol as cryoprotectant. The same cryoprotectant combination gave 90-61% survival of lobster sperm cells in the present study. Much higher percent (95%) viability was shown in *Scylla serrata*, after 30 days of storage in glycerol and DMSO along with the addition of trehalose [5]. Similar results on survivability were reported by Lahnsteiner et al. [19] in rainbow trout, brown trout, lake trout, brook trout and white fish semen. McAndrew [20] described that tilapia spermatozoa protected with 12.5% methanol in fish ringer and held in a vapour phase liquid nitrogen, remained viable for at least 13 months. But, Pillai et al. [21] are of the view that methanol is toxic when added as diluent to store prawn larvae. However, in the present study lobster spermatozoa cryopreserved in DMSO with glucose along with methanol gave higher survival (85-56%) than without methanol (83-50%). Diwan and Nandakumar [22] cryopreserved the sperm cells of *Liza parsia* in DMSO with glucose to get a survival rate up to 50%.

Glycerol and sucrose have been reported to be toxic by Renard and Cochard [23] while cryopreserving *Crassostria gigas* larvae. According to Subramoniam and Newton [24] glycerol above 5% v/v was extremely toxic to the embryos of penaeid prawn, *Penaeus indicus*. Among the different cryoprotectants used in the present study, the combination of glycerol and glucose gave minimum survival (74-47%) of lobster (*P. homarus*) spermatozoa.

Organic constituents decreased in semen after cryopreservation. Maximum decrease of total protein, total free sugar and total lipid were recorded when glycerol and glucose were used as cryoprotectant, while minimum decrease was observed with DMSO and glycerol combination. The decrease was intermediate for the cryoprotectant combinations such as DMSO and glucose with and without methanol. It is interesting to note that minimum organic

components were recorded for glycerol and glucose combination where the survivability of sperm cells was minimum and highest organic constituents recorded for DMSO and glycerol were the survivability was maximum. This could possibly be due to the best cryoprotection capacity of DMSO and glycerol combination, which permitted better inactivation of the sperm cells and reduce metabolic activities thereby reducing the utilization of the biochemical components. Glycerol and glucose being poor cryoprotectants for lobster (*P. homarus*) sperm cells, allowed higher metabolic activity thereby leading to higher utilization of organic constituents. The supporting evidence is from Yoo et al. [25] who have shown the loss of protein fraction from the sperm cells after cryopreservation (*Salmo salar* L.). Similarly Jeyalectumie and Subramoniam [5] suggested the reduction of free sugar and lipid in the cryopreserved seminal plasma of crab (*Scylla serrata*), suggesting the continued metabolic activity of sperm cells by exhausting free sugar and lipid as substrate. However, decreased level of lipid in the post-thawed bovine spermatozoa reflects the cellular destruction during freezing and thawing process as per Pickett and Komarek [26]

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