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Studies on Long- Term Storage of Sperm of a Marine Ornamental Fish, *Istiblennius edentulus* (Perciformes : Blennidae)

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Detailed study was conducted on the effect of cryopreservation on spermatozoa of a marine ornamental fish of the family Blennidae. The long-term preservation of spermatozoa of Istiblennius edentulus was conducted with 5 different extenders in combination with 5%, 7.5% and 10% concentrations of DMSO, Methanol and Glycerol as cryoprotectants. The best post thaw motility was observed with Alsever's solution as extender and 5% Glycerol as cryoprotectant (88.12% motility and 89.19% viability).

Key words : Marine Ornamental Fish, Blennidae, *Istiblennius edentulus*, Spermatozoa, Cryopreservation

With the development of cryopreservation technique, extensive studies are being carried out worldwide for the preservation of milt in fishes which can revolutionise seed production technology of several commercially important species. Most of the studies on this topic are restricted to the European freshwater fishes. Literature on cryopreservation of spermatozoa of tropical marine fishes is limited. The pioneering studies of Blaxter (1953, 1955) and Mounib *et. al.* (1968) paved way for the future investigations on cryopreservation of spermatozoa of tropical fishes. Since then, long-term preservation of milt has been standardized for a large number of fishes. Jenson & Aldredice (1984) concluded that gametes of fishes respond to cryopreservation depending on the species and condition of storage. Cryopreservation of spermatozoa of marine ornamental fishes of Indian origion is limited (Sivaprasad *et. al.*, 2002, 2004).

In the present study five different extenders and three cryoprotectants in different concentrations were used for cryopreservation of spermatozoa of an important marine ornamental fish, *Istiblennius edentulus*.

Materials and Methods

Live specimens of *Istiblennius edentulus* were collected by hand picking from the rocky area of Vizhinjam coast, Kerala. Male specimens of average size (60mm length and7.5 g weight) were selected for the present study.

The milt was collected from ripe males of *I. edentulus*. The fishes were anaesthetized immediately after collection, they were then dissected and the testes were removed. The milt was drawn into 1.5ml Eppendorff tubes. Care was taken to ensure that faeces, blood, urine,

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water and mucus did not contaminate the semen. All observations were made within three hours of collection.

The extenders used for cryopreservation of spermatozoa should mimic the composition and chemistry of seminal plasma. A variety of artificial extender systems were examined. These extender systems were selected based on their beneficial effects observed in various marine fishes. The majority of study was done using five artificial extender systems. The name and composition of the artificial extender system used for the present study is given in Table 1.

Table 1. Composition of the extenders used for long term preservation of spermatozoa of fishes

Extender		Composition	
Marine Fish Ringer		NaCl	1.35g.
		KCl	0.06g.
		$NaHCO_3$	0.02g.
		$MgCl_2$	0.35g.
		Distilled Water	100ml.
2.	Alsever's Solution	Sodium citrate	0.08%.
		Dextrose	2.05%.
		NaCl	0.4%.
		Distilled Water	100ml.
3.	$DCSB_4$	NaCl	9.75g.
		$MgSO_4$	0.125g.
		$CaCl_2 2H_2O$	0.125g.
		Glycine	3.125g.
		Tris HCl	1.2g.
		Distilled Water	1 Litre.
4	$DCSD_2$	NaCl	9.75g.
		KCl	0.5g.
		Glycine	3.125g.
		$NaHCO_3$	1.1g.
		Distilled Water	1 Litre.
5	HFX # 1	NaCl	5.16g.
		KCl	1.64g.
		$CaCl_2$	0.143g.
		$NaHCO_3$	1g.
		$NaH_2PO_4H_2O$	0.410g.
		$MgSO_4 7H_2O$	0.223g.
		Fructose	1g.
		Distilled Water	1 Litre

The dilution ratio of semen to extender used to preserve the spermatozoa was 1:4. The extender was added to the semen and not vice-versa. After an equilibration time of 10 minutes the samples were preserved. The extender was added to the semen at a controlled rate. 4 ml of extender was added to the semen over an approximate 10 minutes period as in the following sequence.

1$^{s.t}$ addition... 0.25 ml of extender added and mixed 2 minutes equilibration period

2nd addition... 0.50ml of extender added and mixed 2 minutes equilibration period

3rd addition... 0.75 ml of extender added and mixed 2 minutes equilibration period

4thaddition... 1ml of extender added and mixed 2 minutes equilibration period

5th addition... 1.5 ml of extender added and mixed 2 minutes equilibration period

Three replications in each experiment were maintained for each species of fishes and samples preserved.

Cryoprotectants like Glycerol, Methanol and Dimethyl sulfoxide (DMSO) were used for the present study. Concentrations of 5%, 7.5% and 10% of each of these cryoprotectants were added to the extended semen (v/v).

After giving an additional 10 minutes equilibration period, the specific samples taken in the Eppendoff tubes were slowly lowered inside the cryocan and placed directly into liquid nitrogen (-196°C) and stored for one year.

On completion of storage period, The preserved samples were taken out and thawed by immersion in a water bath of temperature 30°C (warming rate of approximately 10°C/ minute) and parameters like percentage of motile spermatozoa and spermatozoan viability were observed.

Results and Discussion

The results showed that the spermatozoa of *I. edentulus* yielded higher percentages of motility and viability even after one year of cryopreservation. Alsever's solution (Extender

Table 2. Storage of milt of *I. edentulus* with extender 1 in different % and types of cryoprotectants for one year

Name of Cryoprotectants	% of Cryoprotectants	% of motile spermatozoa		Viability	
		MEAN	SEM	MEAN	SEM
DMSO	5	59.85	± 2.08	62.22	± 3.01
	7.5	69.55	± 3.11	70.09	± 2.80
	10	67.80	± 2.09	68.01	± 3.21
Methanol	5	58.26	± 2.11	59.97	± 3.22
	7.5	57.60	± 0.89	60.79	± 3.11
	10	66.00	± 2.01	67.69	± 2.33
Glycerol	5	60.86	± 2.82	61.09	± 3.11
	7.5	62.12	± 3.72	63.60	± 2.79
	10	66.34	± 3.66	67.44	± 4.22

Table 3. Storage of milt of *I. edentulus* with extender 2 in different % and type of cryoprotectants for one year.

Name of Cryoprotectants	% of Cryoprotectants	% of motile spermatozoa		Viability	
		MEAN	SEM	MEAN	SEM
DMSO	5	65.29	± 3.21	66.79	± 4.09
	7.5	69.15	± 2.49	70.89	± 1.89
	10	71.01	± 1.98	73.87	± 2.38
Methanol	5	68.36	± 3.23	69.45	± 3.28
	7.5	69.45	± 2.56	70.67	± 2.69
	10	66.00	± 3.61	68.79	± 1.88
Glycerol	5	88.12	± 3.76	89.19	± 2.52
	7.5	78.18	± 3.56	70.77	± 3.09
	10	75.89	± 2.45	76.53	± 3.59

Table 4. Storage of milt of *I. edentulus* with extender 3 in different % and type of cryoprotectants for one year.

Name of Cryoprotectants	% of Cryoprotectants	% of motile spermatozoa		Viability	
		MEAN	SEM	MEAN	SEM
DMSO	5	64.82	± 1.89	66.13	± 2.81
	7.5	64.99	± 3.17	64.89	± 2.36
	10	63.29	± 2.26	64.06	± 2.59
Methanol	5	60.19	± 3.15	59.30	± 3.21
	7.5	66.00	± 2.14	67.79	± 3.56
	10	62.77	± 1.27	63.39	± 3.29
Glycerol	5	57.43	± 3.10	58.11	± 2.14
	7.5	56.45	± 3.61	57.67	± 3.29
	10	53.99	± 3.32	56.06	± 3.09

Table 5. Storage of milt of *I. edentulus* with extender 4 in different % and type of cryoprotectants for one year.

Name of Cryoprotectants	% of Cryoprotectants	% of motile spermatozoa		Viability	
		MEAN	SEM	MEAN	SEM
DMSO	5	73.80	± 1.49	74.18	± 2.66
	7.5	75.79	± 3.10	77.02	± 2.49
	10	67.01	± 3.02	78.00	± 2.89
Methanol	5	61.28	± 3.66	62.34	± 2.69
	7.5	64.60	± 2.48	66.79	± 2.58
	10	66.99	± 3.46	67.49	± 3.66
Glycerol	5	76.96	± 1.98	78.00	± 2.46
	7.5	72.90	± 2.23	72.99	± 3.55
	10	79.59	± 2.39	79.84	± 3.33

Table 6. Storage of milt of *I. edentulus* with extender 5 in different % and type of cryoprotectants for one year.

Name of Cryoprotectants	% of Cryoprotectants	% of motile spermatozoa		Viability	
		MEAN	SEM	MEAN	SEM
DMSO	5	71.92	± 1.89	72.00	± 2.99
	7.5	67.66	± 2.10	68.12	± 1.88
	10	55.79	± 3.11	55.93	± 1.93
Methanol	5	65.70	± 2.79	66.01	± 2.66
	7.5	57.69	± 3.13	58.55	± 3.12
	10	58.00	± 2.79	59.62	± 3.63
Glycerol	5	69.16	± 2.33	69.09	± 2.67
	7.5	63.92	± 4.01	63.06	± 3.86
	10	62.22	± 3.00	63.44	± 2.09

2) with 5% of glycerol was found ideal as the extender cryoprotectant system for the Blennidae. In Extender 2 with 5% glycerol, the motility of spermatozoa was reduced from 100% (0-day) to 88.12% after one year of storage in liquid nitrogen, whereas the viability of spermatozoa was reduced to 89.19% from 100%. Tables 2-6 represent the percentage motility and viability of spermatozoa of *I. edentulous* in different extender cryoprotectant systems after one year of storage.

The results of the experiments indicated that the spermatozoa of *I. edentulus* could

withstand the cryopreservation for 365 days and yielded high percentages of motility and viability. Effectiveness of glycerol in cryopreservation was observed by Hoyle & Idler (1968) and Truscott & Idler (1969). The success of extender cryoprotectant system varies with species. Experiments of Withler & Lim (1982) on the long - term storage of spermatozoa of *Epinephelus tauvina* found that DMSO (10%) maintained the best motility and viability. Kerby (1983) noticed the immotile spermatozoa in DMSO and motile spermatozoa in glycerol.

The present observations are in agreement with that of Gwo *et al.* (1991) and Piironen (1993). Glycerol in concentrations from 5% to 10% gave best results. Suitability of glycerol was also confirmed in the preservation of spermatozoa of *Crassostrea* sp. (Yankson & Moyse, 1991), *Trichoserus vulpecule* (Rodger *et. al.*, 1991), *Tetraselmis* sp. (Day & Fenwick, 1993) and *Thalassoma lunare* (Sivaprasad *et. al.*, 2004). Kumar *et. al.*, (1994) reported that glycerol is the best cryoprotectant for the storange of cattle milt. The present observation also supports the above findings regarding the suitability of glycerol as an ideal cryoprotectant. According to Gwo *et. al.*, (1991) the response of spermatozoa to different cryoprotectants is strictly species specific and there is no universal cryoprotectant.

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