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Effect of extenders, cryoprotectants and dilution ratios on the spermatological properties of cryopreserved milt of cobia *Rachycentron canadum*

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Abstract

Cobia (*Rachycentron canadum*) culture depends on the availability of seeds which is affected by asynchronous spawning and brooders availability. The possible solution for these problems is the use of cryopreserved, high quality gametes from selected individuals. Therefore, in the present study, the effect of different extenders, different concentrations of cryoprotectants and dilution ratios on the spermatological properties of cryopreserved cobia milt was analysed. Milt was collected from *R. canadum* brooders and diluted with Marine Fish Ringer's Solution (MFRS) and 0.85% physiological saline (PS) as extenders and dimethyl sulphoxide (DMSO) as cryoprotectant at three different concentrations (5, 10 and 15%) at three dilution ratios such as 1:5, 1:10 and 1:15. Motility duration, motility pattern, motility score and percentage of live and dead cells were analysed. The milt was rapidly frozen employing liquid nitrogen vapour for 10 min. The milt sample diluted with PS and 10% DMSO at 1:5 dilution ratio exhibited highest post-thaw motility parameters.

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Introduction

Global capture fisheries production reached 96.4 million t in 2018, the highest level ever recorded (FAO, 2020). Since most of the capture fisheries resources worldwide are considered overexploited, there is a pressing need to meet the growing demand for fish driven by population growth, rising incomes and increasing urbanisation. Development of sustainable aquaculture and improvements in the post-harvest sector are needed to reduce losses which will help to maintain fish supply and the contribution of fish to development (Finegold, 2009). Reducing exploitation of marine fishes through capture and farming of the same through mariculture is the need of the hour.

Cobia (*Rachycentron canadum*) is one of the important marine finfish species which have very high potential for aquaculture (Nazar *et al.,* 2019). It is a fast growing fish belonging to the monotypic family Rachycentridae. It is distributed throughout the world, except in the central and eastern Pacific (Shaffer and Nakamura, 1989). It is a pelagic species and mostly occur in the water column of the open ocean from the surface to a depth of 1200 m (Shaffer and Nakamura 1989). Cobia stocks are being exploited at rates beyond sustainable yields and hence, there is a need for development of techniques related to commercial culture of cobia (Cake *et al.,* 1989).

Attributes like fast growth rate, adaptability for captive breeding, low cost of production, good meat quality and high market demand especially for *sashimi* industry, makes cobia an excellent species for aquaculture. They can also grow in low saline water and it has been reported that up to 15 ppt, the growth and survival is comparable to that in seawater (Nazar *et al.,* 2019). These findings indicate the potential for the development of cobia aquaculture in India (Nazar *et al.,* 2019).

The major problem faced in culturing cobia is availability of seeds which is affected by asynchronous spawning behaviour of this

species. Maintaining male and female brooders in the farm require long time and high input cost. Maintaining only female brooders in the farm can reduce the maintenance cost of male brooders which can be achieved by cryopreservation of spermatozoa. Cryopreservation is a technique that allows sperm to be stored for an indefinite period of time (Sarvi *et al.,* 2006; Martínez-Paramo *et al.,* 2017). To date milt of over 200 species of freshwater and marine fish have been cryopreserved (Lakra, 1993; Rana, 1995; Blesbois and Labbe 2003; Hiemstra *et al.,* 2005).

The success of cryopreservation depends not only on preserving the motility of the spermatozoon but on maintaining its metabolic functions (Ciereszko and Dabrowski, 1996). Freeze-thaw procedures can induce irreversible and lethal damage to sperm, impairing spermatozoon motility (Linhart *et al.,* 2000), viability (Li *et al.,* 2010) and acrosome structure (Psenicka *et al.,* 2008) and directly impact successful fertilization and embryo development (Suquet *et al.,* 1998). The most critical factors that need be optimised for the successful preservation of fish gametes are the composition of the extenders, cryoprotectants, equilibration time, rate of freezing and thawing temperature (Lahnsteiner, 2000). Hence the present study analysed the effect of cryopreservation on the spermatological properties of *R. canadum* spermatozoa and also envisaged to standardise the cryopreservation protocol for *R. canadum* spermatozoa for long term storage.

Materials and methods

Experimental fish

R. canadum reared and grown in the open sea cages, at the Marine Finfish Hatchery Project, Rajiv Gandhi Centre for Aquaculture (RGCA), Muttom, Kanyakumari District, Tamil Nadu, India served as the milt donors for the present study. The fishes were collected from open sea cages using hand net and were transferred to FRP tank of 10,000 l capacity. The fishes were anesthetised in the FRP tank. Commercial anaesthetic "AQUI-S" which has an active ingredient of 540 g l^{-1} isoeugenol was added in water at 15 mg l^{-1}. Upon addition of anaesthetic, foam was noticed in the water and the fishes were found loosing balance in 5-10 min. After anesthetisation, cannulation was done following Caylor *et al.* (1994). The fish was turned upside down and a canula of 1.2 mm diameter was inserted through the genital opening to sample the gonad material. Fishes which released eggs during cannulation were identified as females whereas, fishes which released milt were identified as males.

Milt collection

Male brooders weighing approximately 115 cm length and 17.5 kg weight was used for milt collection. The milt was collected by cannulation procedure (Caylor *et al.,* 1994) from the anesthetised brooders as prescribed by Umaa Rani *et al.* (2016). Milt was collected from 5 brooders, pooled together and transferred to pre-labelled sterile graduated vials (2.5 ml) with sufficient air space for oxygenation of sperms as suggested by Rani and Munuswamy (2014). The vials containing milt samples were kept refrigerated on gel ice (6-10°C) as suggested by Asturiano *et al.* (2004) and transported from the collection site to the laboratory.

Experimental design

The study consisted of three independent trials. In trial 1, the effect of different extenders on the sperm motility was evaluated. The aim of Trial 2 was to evaluate the effect of different concentrations of cryoprotectant on the sperm motility and Trial 3 was performed to assess the effect of dilution ratios on the milt quality.

Effect of extenders on sperm motility

Two extenders such as Marine Ringers Solution (MFRS) (1.35 g NaCl, 0.06 g KCl, 0.02 g $NaHCO_3$, 0.025 g $CaCl_2$, 0.035 g $MgCl_2$ per 100 ml of distilled water) (Chao *et al.,* 1975) and 0.85% Physiological Saline (PS) (0.085 g NaCl per 100 ml of distilled water) (Betsy and Kumar, 2015) were used for cryopreservation of *R. canadum* spermatozoa. All chemicals were of analytical grade purchased from Merck, Germany. Dimethyl Sulphoxide (DMSO) was used as cryoprotectant at 10% (v/v) concentration. The milt was diluted with these cryodiluents at 1:10 ratio and equilibrated for 10 min at 4°C in the cold handling unit as suggested by Rafiquzzaman *et al.* (2007). The equilibrated milt was loaded into 0.25 ml straws and were sealed using polymer powder (IMV, France). The straws were arranged horizontally on floating rack (13×13 cm) and was allowed to float in liquid nitrogen held in a Styrofoam box (Betsy and Kumar, 2019). The distance between the straws and liquid nitrogen (LN_2) was maintained at 3 cm for a uniform freezing effect (Boryshpolets *et al.,* 2017; Betsy and Kumar, 2019). The milt was rapidly frozen by LN_2 vapour for 10 min as recommended by Asturiano *et al.* (2004) before plunging it in LN_2. The cryopreserved spermatozoa was analysed once in 10 days for a period of 30 days after thawing of the straws at 30°C for 30 s. The thawed milt was observed for the same parameters of the pre-frozen milt and the data were recorded.

Effect of cryoprotectant concentration on sperm motility

The best performing extender was selected from previous trial. The milt was diluted with DMSO as cryoprotectant at 5, 10 and 15% (v/v) concentrations at dilution ratio of 1:10 and the milt was cryopreserved following the same procedure as mentioned above.

Effect of dilution ratios on sperm motility

Based on the previous trials, the best performing extender and cryoprotectant concentration was selected for this trail. Freshly collected milt was diluted at three dilution ratios *viz.,* 1:5, 1:10 and 1:15 and the milt was cryopreserved following the same procedure as mentioned above.

Assessment of spermatological properties

Spermatological parameters such as sperm motility, motility pattern, motility score and percentage of live cells were analysed in raw milt. Duration of motility was estimated as the time elapsing from water addition to the decrease in motility to about half of the original value using stopwatch having sensitivity of 0.01 s (Babiak *et al.,* 1995). Motility score was assigned to each sample (Betsy

and Kumar, 2014). The percentage of motility of the spermatozoa was evaluated microscopically following Sarder *et al.* (2011). The percentage of live and dead spermatozoa was assessed using Eosin-Nigrosin stain as described by Chutia *et al.* (1998).

Statistical analysis

All measurements were taken in triplicates and the Mean±SD was noted. The collected data was then tabulated and analysed by one-way analysis of variance (ANOVA) using SPSS 22.0 software for Windows. The statistical significance for all data were compared at 5% probability level and mean values between different experimental treatments were compared by contrast analysis in Duncan's multiple range tests.

Results and discussion

Spermatological parameters of raw milt

The motility duration of fresh spermatozoa of *R. canadum* was 58 s. This observation is in parallel to the finding in kelp grouper (Miyaki *et al.,* 2005) for which the average motility duration was reported to be 60 s. Reports on the motility duration of fresh, undiluted milt of other marine species could not be traced out. However, the fresh diluted milt of sterlet (*Acipenser ruthenus*) had 89±3% motility as reported by Xin *et al.* (2018). This reveals that the marine fishes may have a motility duration ranging from 50 s to 100 s, which needs to be further investigated. Most of the spermatozoa exhibited forward and circular movement which could be scored at 9.

Influence of extenders on cryopreserved spermatozoa

When PS was used as extender for cryopreserving *R. canadum* milt, the highest mean post-thaw motility duration of 53.31±0.22 s was found on 1st day which decreased to mean post-thaw motility duration of 39.19±0.13 s on 30th day of the experiment (Table 1). When milt was diluted with MFRS, the highest mean post-thaw motility duration obtained was 52.31±0.01 s which decreased to 37.23±0.06 s on 30th day (Table 2). The values were statistically significant between the two extenders when analysed using one-way ANOVA ($p>0.05$).

McAndrew *et al.* (1993) stated that for cryopreservation of teleost spermatozoa, the chemical composition of extender media varied greatly with species and protocol adopted. The sperm motility could be prevented by potassium in the seminal plasma (Scheuring, 1924; Schlenk, 1938). Sodium, calcium and magnesium reduced the inhibiting action of potassium on motility of spermatozoa (Scheuring, 1924).

MFRS used in the present study had 231 mM NaCl and 8 mM KCl whereas PS had only 145 mM NaCl. According to the observations by Ciereszko *et al***.** (2002) in the sea lamprey, just after initiation of movement, motility was inhibited by concentrations of NaCl and KCl higher than 40 mM whereas, at 2 min after activation, motility was inhibited at NaCl and KCl concentrations of over 20 mM. Hence it can be inferred that MFRS which had higher mM of NaCl compared to PS exhibited lower motility duration. Although PS had 145 mM NaCl, it was lower when compared to 231 mM NaCl present in MFRS. It must be also noted that MFRS had some quantity of KCl which is also inhibitory in nature. Therefore this may be the reason for higher motility duration recorded with PS.

Table 1. Mean motility duration (s) of *R. canadum* spermatozoa cryopreserved with different extenders

Extender	Days of cryopreservation			
	Initial	10	20	30
FWFS	52.31±0.01^{b}	44.97±0.03^{b}	40.03±0.03^{b}	37.23±0.06^{b}
PS	53.31±0.22^{a}	48.03±0.03^{a}	42.37±0.03^{a}	39.19±0.13^{a}

Data expressed as Mean±SE (n=10, r=3); Mean values in the same column with different superscript differ significantly ($p<0.05$). One way ANOVA was used following Duncan's multiple range test in SPSS- 22.0.

Table 2. Mean motility duration (s) of *R. canadum* spermatozoa cryopreserved with DMSO at 3 different concentrations

DMSO concentrations (%)	Days of cryopreservation			
	Initial	10	20	30
5	50.34±0.01^{c}	42.35±0.11^{c}	40.61±0.03^{c}	36.04±0.04^{c}
10	55.27±0.09^{a}	49.19±0.11^{a}	43.66±0.01^{a}	39.19±0.13^{a}
15	53.53±0.11^{b}	46.12±0.66^{b}	42.37±0.03^{b}	37.23±0.06^{b}

Data expressed as Mean±SE (n=10, r=3); Mean values in the same column with different superscripts differ significantly ($p<0.05$). One way ANOVA was used following Duncan's multiple range test in SPSS- 22.0.

When PS was used as a diluent for cryopreservation of *R. canadum* milt, the spermatozoa exhibited only forward and circular movement throughout the experiment. This was similar to the observations made by Balamurugan and Munuswamy (2017) in grey mullet who reported fast swimming spermatozoa when milt was diluted with V2E extender that contained 128.5 mM of NaCl and 5.10 mM of KCl.

The spermatozoa exhibited weak vibratory movement at the initial day of the experiment when MFRS was used and at the end of the experiment, few spermatozoa showed weak oscillatory movement while most of the spermatozoa became immotile. When the results of the present study is compared with the report of Balamurugan and Munuswamy (2017) in grey mullet, it can be understood that the optimum motility properties of marine fish spermatozoa is obtained only when NaCl and KCl are present at concentrations lower than 130 and 5 mM respectively.

The motility score varied with respect to the type of extender used and the value decreased at the end of the cryopreservation period for both the diluents. When *R. canadum* milt was cryopreserved with PS, the motility score fluctuated during the experiment. On initial day motility score was 9, which reduced during the cryopreservation period and reached 6 at the end of the experiment. Similarly, after cryopreservation of *R. canadum* milt with MFRS as extender, the motility score dropped from 2 on 1st day to 0.5 on 30th day.

The highest sperm motility grade of 3.0±0.0 (50%–80% sperm movement) was obtained in grey mullet by diluting the sperm with V2E extender (Balamurugan and Munuswamy, 2017). In the present study, PS gave the highest post-thaw motility score of 9 which dropped to the maximum of 6. Although the present study could not be directly compared with the study of Balamurugan and Munuswamy (2017) as it is with grey mullet, the values obtained in

the reduction in the motility score was higher than that reported by them. It can be further attributed to the absence of motility inhibitory compounds like KCl in PS as pointed out by Schlenk (1938).

Cryopreservation of *R. canadum* spermatozoa with PS gave the highest percentage of live cells. At the start of the experiment, 89% of the spermatozoa were found alive, which was the highest and reduced to 61% on 30th day of cryopreservation. The highest percentage of live cells observed in milt diluted with MFRS was 79% which decreased to 49% at the last day of cryopreservation. This may be attributed to the fact that since PS had only 145 mM NaCl and no KCl, it would have better retained the spermatozoa and gave higher number of live spermatozoa when compared with MFRS.

Influence of DMSO on cryopreserved spermatozoa

When DMSO was added at 5% concentration, the highest mean post-thaw motility duration noticed was 50.34±0.01 s which dropped to 36.04±0.04 s at the end of the cryopreservation (Table 2). The highest mean post-thaw motility duration recorded when *R. canadum* milt was cryopreserved with 10% DMSO was 55.27±0.09 s which got reduced to 39.19±0.13 s at the end of the experiment. When milt was cryopreserved with DMSO at 15% concentration, the mean post-thaw motility duration obtained at the end of the experiment was 37.23±0.06 s. The values between different DMSO concentrations were statistically significant ($p>0.05$) when analysed using one way ANOVA. The result was in accordance with Caylor *et al*. (1994) who achieved satisfactory result with 10% DMSO for the cryopreservation of *R. canadum* spermatozoa.

Studies have reported that 10% DMSO yielded the highest post-thaw motility in marine fish species such as Japanese bitterling (Ohta *et al.,* 2001) red snapper (Riley *et al.,* 2004), mangrove red snapper (Vuthiphandchai *et al.,* 2009), dusky grouper (Cabrita *et al.*, 2009), seven-band grouper (Koh *et al.,* 2010), red-spotted grouper (He *et al.,* 2011), Atlantic halibut (Ding *et al.,* 2011), mutton snapper (Sanches *et al*., 2013), lane snapper (Gaitan-Espitia *et al.,* 2013) and brown-marbled grouper (Yang *et al.,* 2020).

Stoss and Holtz (1983) and Leung (1987) observed that DMSO led to toxic effects on spermatozoa if used in higher concentrations, however, DMSO at concentration of 5-20% is ideal for cryopreservation of fish sperm. DMSO has fast penetration rate and interacts with membrane phospholipids (Anchordoguy *et al.,* 1987). Hence optimum DMSO concentration often range from 10 to 20% for cryopreservation of marine fish spermatozoa (Gwo, 2000). This observation held good in the present study. This may be because DMSO allows a rapid balance between the intracellular and extracellular fluid concentrations (Ciereszko *et al.,* 1993) and when DMSO was used at lower concentrations it could lead to poor result since the spermatozoa die as a result of intracellular ice formation (Ohta *et al.,* 2001). This is due to the lower concentration of DMSO which would be insufficient to force water out of the cells (Ohta *et al.,* 2001).

When DMSO was used at 5 and 10% concentration for cryopreservation of *R. canadum* milt, it did not have any influence on the motility pattern. Similarly, when *R. canadum* milt was cryopreserved with DMSO, no change in motility score was noticed. However, the score was influenced only by the diluent used and the cryopreservation period.

When 5% DMSO was used for cryopreservation, highest percentage of live cells obtained was 84% which gradually reduced to 54% on 30th day. The lowest percentage of live cells noticed with 5% DMSO was 73% which decreased to 46% at the end of the experiment. When *R. canadum* milt was cryopreserved with 10% DMSO as cryoprotectant, the highest percentage of live cells observed was 89% which declined to 61% at the end of the experiment. The lowest percentage of live cells recorded at 10% DMSO was 72% which dropped to 42% on 30th day. Formation of intracellular ice at 5% DMSO may be a possible reason for the comparatively lower percentage of live cells with 5% DMSO (Ohta *et al.,* 2001).

Influence of dilution ratio on cryopreserved spermatozoa

The highest mean post-thaw motility duration observed on the first day of experiment was 41.37±0.09 s, 39.34±0.11 s and 35.11±0.06 s when milt was diluted at 1:5, 1:10 and 1:15 ratio, respectively (Table 3). These values decreased to 31.04±0.04 s, 25.23±0.06 s and 24.26±0.21 s at the end of the experiment at 1:5, 1:10 and 1:15 dilution ratio, respectively and the values were statistically significant between the three dilution ratios ($p<0.05$) when analysed using one-way ANOVA.

The observation on the post-thaw motility in the present study is in accordance with the reports of Dreanno *et al.* (1997) who stated that dilution ratio higher than 1:10, reduced cryopreservation efficiency in turbot spermatozoa. However, in European eel, the dilution limit was found to be 1:5 (Marco-Jimenez *et al.,* 2006). This may be because of dilution of protective substances present in seminal fluid such as protein, glucose and ions (Marco-Jimenez *et al.,* 2006; Mommens *et al.,* 2008) that could impair the spermatozoa if it exceed a limit.

Several studies revealed that high dilution ratio reduces the motility and viability of frozen sperm (Cabrita *et al.,* 2005) because of the dilution of several proteins (Suquet *et al.,* 2000) and anti-oxidative stress compounds (Cabrita *et al.,* 2011) present in the seminal plasma that protect sperm viability during the freezing and thawing processes. This may be the reason for low motility duration when *R. canadum* milt was diluted at 1:15 ratio.

Dilution ratios used in the present study such as 1:5, 1:10 and 1:15 for cryopreserving *R. canadum* milt had showed no difference in the motility pattern among the dilution ratios. When milt sample was diluted at 1:5, 1:10 and 1:15 dilution ratios, no change in motility score was noticed. When milt was diluted at 1:5 ratio, the highest percentage of live cells observed on the initial day of experiment was 89% which decreased to 61% on 30th day. The highest percentage of live spermatozoa noted at 1:10 dilution

Table 3. Mean motility duration (s) of *R. canadum* spermatozoa cryopreserved at 3 dilution ratios

Dilution ratio	Days of cryopreservation			
	Initial	10	20	30
1:5	41.37±0.09^{a}	38.34±0.06^{a}	35.27±0.06^{a}	31.04±0.04^{a}
1:10	39.34±0.11^{b}	35.16±0.03^{b}	31.48±0.09^{b}	25.23±0.06^{b}
1:15	35.11±0.06^{c}	32.35±0.11^{c}	28.48±0.09^{c}	24.26±0.21^{c}

Data expressed as Mean±SE (n=10, r=3); Mean values in the same column with different superscripts differ significantly ($p<0.05$). One way ANOVA was used following Duncan's multiple range test in SPSS- 22.0.

ratio was 82% which decreased to 53% on 30th day of experiment. When the milt sample was diluted at the ratio of 1:15, the highest percentage of live spermatozoa recorded was 78% which dropped to 47% on 30th day. The decrease in the presence of live cells during the cryopreservation in the present study is in accordance with Horokhovatskyi (2018) who reported that in cryopreserved samples of sterlet (*A. ruthenus*) spermatozoa, the percentage of live cells decreased from 96% to 70% when diluted at 1:50 dilution ratio.

This is the first study that has attempted to standardise the cryopreservation protocol for *R. canadum* milt. From the present study, it can be concluded that milt of *R. canadum* when cryopreserved with 0.85% Physiological Saline solution and 10% DMSO diluted at 1:5 dilution ratio gave better results in terms of spermatological properties such as higher motility duration, motility score, motility pattern and percentage of live cells. Further investigations need to be undndertaken to validate the effect of cryopreserved milt on the fertilisation success of cobia.

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