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Technical note

Cryopreservation of Brazilian flounder (*Paralichthys orbignyanus*) sperm

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Abstract

The Brazilian flounder, *Paralichthys orbignyanus*, is being considered for aquaculture due to its high demand and market price. Reproduction and larviculture studies have demonstrated the feasibility of massive fingerling production, and techniques that prolong life and increase gamete viability can assist in the culture development of this species. The aim of this study was to evaluate the efficiency of two different cryosolutions for cryopreservation of Brazilian flounder semen in order to improve broodstock management and consequently augment the potential for its culture. Two different cryosolutions were tested: a) glycerol–saline: glycerol solution (12% or 1.65 M) along a saline-based diluent (423 mM NaCl, 9 mM KCl, 9.25 mM $CaCl_2.2H_2O$, 22.92 mM $MgCl_2.6H_2O$, 25.5 mM $MgSO_4.7H_2O$ and 2.15 mM $NaHCO_3$; pH 8.2; osmolality 900 mOsmol/kg); and b) DMSO–sucrose: DMSO solution (10% or 1.40 M) along a sucrose-based diluent (110 mM Sucrose, 100 mM $KHCO_3$ and 10 mM Tris-Cl; pH 8.2; osmolality 335 mOsmol/kg). Cryopreservation was made without equilibration time. First, 250 µl-straws were placed 6 cm above the surface of liquid nitrogen for 10 min, then they were maintained for 5 min on the surface of liquid nitrogen (1 cm) before being plunged into liquid nitrogen. The quality of cryopreserved sperm was assessed through the percentage of sperm motility and viability, fertilization capacity, hatching and larval viability. Motility was estimated with an arbitrary scale, ranging from 0 to 5. Spermatozoa viability was determined using a LIVE/DEAD® sperm viability kit. Motility of fresh sperm (3.5±0.2) was similar to frozen/thawed sperm with DMSO-sucrose (2.5±0.3) ($P>0.05$). On the other hand, the motility of frozen/thawed sperm with glycerol-saline (1.3±0.4) was lower than the other two treatments ($P<0.05$). No difference was found in the percentage of live spermatozoa post-thawed between DMSO–sucrose and glycerol–saline solutions ($P<0.05$). However, fresh sperm had a higher percentage of live spermatozoa than post-thawed sperm with glycerol-saline ($P<0.05$). Sperm motility was positively correlated with the percentage of live spermatozoa (Adjusted $R^2=0.61$, $n=13$). No difference was found for fertilization and hatching rates and larvae viability among the three treatments ($P>0.05$). This is the first report on successful cryopreservation of Brazilian flounder sperm. This procedure should improve broodstock management techniques for this species and consequently augment the potential for its culture.

Keywords: Glycerol–saline; DMSO–sucrose; Spermatozoa; *Paralichthys orbignyanus*; Reproduction

1. Introduction

The Brazilian flounder *Paralichthys orbignyanus* inhabits estuarine and coastal waters from Rio de Janeiro (Brazil) to Mar del Plata (Argentina) (Figueiredo and Menezes, 2000). Decreasing landings associated with high demand and market price have stimulated the culture of Brazilian flounder. Tolerance to a wide range of temperatures (Wasielesky et al., 1998), salinities (Sampaio and Bianchini, 2002), as well as high concentrations of nitrogenous compounds (Bianchini et al., 1996) are characteristics that supported the initial studies related to its culture. More recently, studies related to reproduction (Bianchini et al., 2005; Radonic et al., 2007) and larviculture

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(Sampaio et al., 2007) showed the feasibility of fingerling production.

Gamete cryopreservation has been identified as a tool to improve broodstock management and consequently increase the number of juveniles produced from artificial fertilization (Chereguini et al., 2003). However, despite the efforts, cryopreservation of finfish ova and embryos has not been attained yet. So far only sperm cryopreservation has proved to be successful (Chao and Liao, 2001).

Benefits of sperm cryopreservation include: synchronization of gametes availability, use of the total volume of available sperm, reduction of costs related to maintenance of male broodstock, facilitation of gamete transportation, avoid ageing of sperm, and allow for the conservation of genetic variability for domesticated populations and for endangered species (Suquet et al., 2000).

Sperm cryopreservation has been carried out successfully in more than 200 species of fish (Suquet et al., 2000). However, the success rates of these sperm cryopreservation experiences have been highly variable, among and within methods, as well as among individuals of the same species (Billard et al., 1995). Regarding Pleuronectiformes, sperm cryopreservation of several species has been studied, including the plaice *Pleuronectes platessa* (Pullin, 1972), Atlantic halibut *Hippoglossus hippoglossus* (Bolla et al., 1987), yellowtail flounder *Pleuronectes ferrugineus* (Richardson et al., 1999), winter flounder *Pseudopleuronectes americanus* (Rideout et al., 2003), hirame *Paralichthys olivaceus* (Zhang et al., 2003), turbot *Psetta maxima* (Chen et al., 2004) and spotted halibut *Verasper variegates* (Liu et al., 2006). Dimethyl sulfoxide (DMSO), glycerol and propylene glycol have proved effective as cryoprotectants for sperm of one or more species of flatfish listed above.

The aim of this study was to evaluate the efficiency of two different cryosolutions for cryopreservation of Brazilian flounder semen, a saline-based diluent containing glycerol and other sucrose-based diluent containing DMSO as cryoprotector. The quality of cryopreserved sperm was assessed through the evaluation of sperm motility and viability, fertilization capacity, hatching and larval viability.

2. Materials and methods

2.1. Fish

Brazilian flounder broodstock were captured in the middle of the breeding season (January 2007) at Cassino Beach (Southern Brazil, 32° 12′ S-52° 10′ W) and taken to the Laboratory of Mariculture at the Federal University of Rio Grande (FURG). Males (432±96 g; 35±2 cm mean±standard error) were kept in the laboratory no longer than 10 days before sperm was collected. Holding tanks (1000 l) were placed in a room with controlled photoperiod (14 h light/10 h dark). Water temperature was 25±2 °C and salinity 35‰. Water was renewed at least 100% daily.

2.2. Sperm collection

Before sperm collection, fish were anesthetized with benzocaine (50 ppm). In order to avoid urine and fecal contamination, fish were cleaned and blotted dry with a paper towel. Sperm was hand striped and collected in graduated syringes without needle (1 ml) and immediately transferred to 1.5 ml microtubes.

2.3. Sperm characterization

Before cryopreservation, each sperm sample was checked for motility under a microscope. Sperm with motile spermatozoa was discarded, as contamination with urine or seawater might have occurred. Spermatozoa motility was estimated subjectively under an optic microscope (Olympus CX-41, 400×) immediately after activation with seawater (35‰ ≈ 1050 mOsmol/kg). Dilution was equal to 1:50 (1 μl sperm and 49 μl seawater). Motility was estimated with an arbitrary scale, ranging from 0 to 5, where 0 represents no motility; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–90%; and 5, 91–100% of motile spermatozoa (Borges et al., 2005). Only sperm showing motility above 50% (≥3) after seawater activation were used for cryopreservation.

Sperm density was determined using an improved Neubauer Bright line counting chamber. Sperm was fixed in 4% formalin and diluted in distilled water (1:2000). Prior to counting, samples were left undisturbed for about 10 min in the Neubauer chamber, allowing cells to sediment. Cells were counted under a microscope at 400× magnification.

2.4. Sperm cryopreservation

Cryoprotectant and diluent solutions used in this study were:

a) glycerol–saline: glycerol solution (12% or 1.65 M) along a saline-based diluent (423 mM NaCl, 9 mM KCl, 9.25 mM $CaCl_2.2H_2O$, 22.92 mM $MgCl_2.6H_2O$, 25.5 mM $MgSO_4.7H_2O$ and 2.15 mM $NaHCO_3$; pH 8.2; osmolality 900 mOsmol/kg) (Zhang et al., 2003);
b) DMSO–sucrose: DMSO solution (10% or 1.40 M) along a sucrose-based diluent (110 mM Sucrose, 100 mM $KHCO_3$ and 10 mM Tris-Cl; pH 8.2; osmolality 335 mOsmol/kg) (Chen et al., 2004).

Sperm from seven males was individually split in two samples and diluted 1:3 (v/v; sperm: extender+cryoprotectant) in glycerol or DMSO solutions previously cooled at 5 °C. Before cryopreservation, neither cryosolution induced sperm motility. For each male, three straws (250 μl-IMV; France) were filled with each sperm-extender mixture and the ends were sealed with clay. Cryopreservation was made without equilibration time. First, straws were placed 6 cm above the surface of liquid nitrogen for 10 min, then they were maintained for 5 min on the surface of liquid nitrogen (1 cm) before being plunged into liquid nitrogen. This procedure followed the freezing curve described by Chen et al. (2004).

2.5. Post-thaw sperm motility and viability

Post-thaw sperm motility was evaluated 24 h after cryopreservation. Two straws (one with glycerol–saline and another with DMSO–sucrose) of each male were thawed by immersion in a 37 °C water bath for 30 s, and immediately evaluated as previously described.

Spermatozoa viability was determined using a LIVE/DEAD® sperm viability kit (Molecular Probes, USA) which differentially stains live and dead cells with SYBR 14 and propidium iodide dyes, respectively. Sperm (10 μl) was placed in 1.5 ml microtube and diluted in 500 μl BPSE (5 g/l $C_6H_{12}O_6$, 0.34 g/l $MgCl_2$, 0.64 g/l $C_6H_5K_3O_7$, 4.30 g/l $NaC_2H_3O_2$, 8.67 g/l $C_5H_8NO_4Na$, 1.95 g/l $C_6H_{15}NO_6S$, 0.75 g/l KH_2PO_4, and 12.70 g/l K_2HPO_4; and pH 7.5). Each reaction used 1 μl SYBR 14 (20 nM/μl) and 2.5 μl propidium iodide (2.4 mM/μl). Solutions were incubated for 10 min and analyzed in an epifluorescence microscope (Olympus BX 60) (excitation=450 to 480 nm and emission=520 nm). Spermatozoa viability was calculated as the proportion between the number of live cells and the total number of cells counted.

2.6. Evaluation of fertilization, hatching rates, and larvae viability

Fertilizing capacity of sperm cryopreserved for 30 days (DMSO–sucrose and glycerol–saline) was compared to fresh sperm (for details on the experimental design see Table 1). Fresh sperm was collected from different males. Females (1476±212 g; 50±3 cm) were induced to spawn with carp pituitary extract (5 mg/kg), once ovulation was observed, eggs were gently hand stripped into a 500 ml beaker.

Table 1
Experimental design used to evaluate the fertilizing ability of sperm of Brazilian flounder *Paralichthys orbignyanus* cryopreserved with DMSO–sucrose versus glycerol–saline

	DMSO–sucrose					Glycerol–saline				
	♂ 1	♂ 2	♂ 3	♂ 4	♂ 5	♂ 1	♂ 2	♂ 3	♂ 4	♂ 5
♀ 1	x *	x	x	x		x	x	x	x	
♀ 2		x	x	x	x		x	x	x	x
♀ 3	x				x	x				x

Ten fertilizations were carried out for each cryosolution tested, and all females were also fertilized with fresh semen as a control.
*Sperm from male number 1 cryopreserved with DMSO–sucrose was used to fertilize eggs stripped from female number 1.

Sperm to egg ratio was at least 80,000 sperm/egg. Fertilization was accomplished by adding 70 µl sperm of a straw to 2 ml eggs (≈1800 eggs) placed on a Petri dish. Filtered seawater (2 ml) was immediately added and gently mixed to activate the sperm. Eggs were carefully rinsed with seawater 10 min later and transferred to graduated 10 ml test tubes, where the percentage of floating eggs was determined.

Percentage fertilization was measured 2 h later. Approximately 100 floating eggs were placed in an excavated glass slide and observed under a stereoscopic microscope (Wild Heerbrugg, Switzerland, 16×). Eggs reaching the 4–8 blastomere were considered fertilized.

The hatching rate was estimated from samples of 50 floating eggs, placed in beakers filled with 800 ml of seawater (35‰) and kept at 23 °C in an incubator (Tecnal TE-401, Brazil) until hatching (approximately 40 h after fertilization). For each fertilization test, the hatching rate was repeated three times, and it was calculated as the mean of these three values. Hatching rate was expressed as the percentage of hatched larvae in relation to the total number of floating eggs incubated.

After hatching, 30 larvae of each fertilization trial were observed for malformation (curvature in the vertebral column and incorrect fin development). Larval viability (L_v) was calculated as $L_v=(L_n\times H_r)/100$, where L_n is the percentage of normal larvae and H_r is the hatching rate.

2.7. Statistical analyses

Percentage values were arc–sine transformed prior to analysis. One-way ANOVA was used to compare the effects of cryoprotectants on sperm motility, sperm viability, floating eggs rate, fertilization rate, hatching rate and larvae viability. Differences were considered significant at $P<0.05$. Tukey HSD multiple range test comparisons was used if significant differences existed among treatments. Linear regression analysis was carried out to determine the correlation between sperm motility and sperm viability. Data are presented as mean±SEM. All analyses were conducted using software R version 2.4.0 (Vienna, Austria).

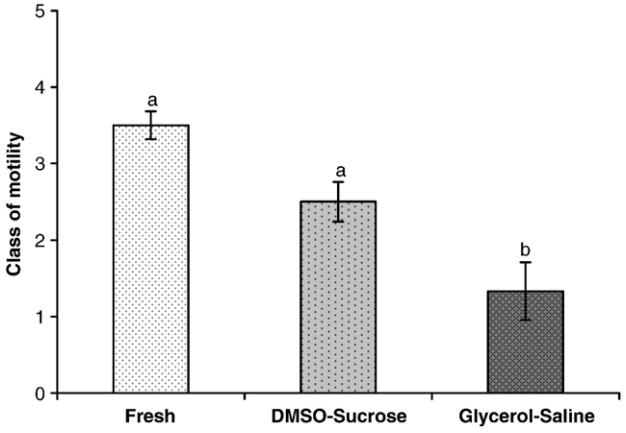

Fig. 1. Motility of fresh and post-thaw sperm of flounder *Paralichthys orbignyanus* ($n=7$). Different letters above each bar indicate significant difference among treatments ($P<0.05$). Data are represented as mean±SEM.

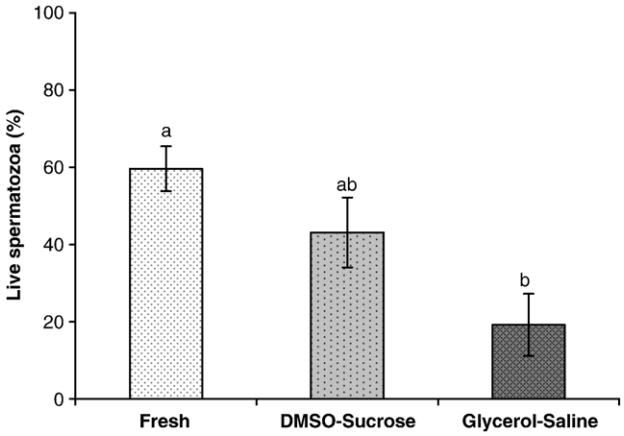

Fig. 2. Percentage of live spermatozoa ($n=7$) determined by differential fluorescent staining for fresh and post-thaw sperm of flounder *Paralichthys orbignyanus*. Different letters above each bar indicate significant difference among treatments ($P<0.05$). Data are represented as mean ± SEM.

3. Results

The average volume of sperm collected from each fish was 475±17 µl. Mean density of spermatozoa was $1.08\pm0.06\times10^{10}$ cells/ml.

Sperm motility in fresh sperm was 3.5±0.2. Spermatozoa cryopreserved with glycerol-saline were motile soon after thawing, even without being activated with seawater. In this case, the motility reached 0.92±0.3. Post-thawed sperm cryopreserved with DMSO-sucrose was not motile before activation with seawater. The motility of frozen–thawed sperm with DMSO-sucrose (2.5±0.3) was significantly higher than that observed for sperm cryopreserved with glycerol-saline (1.3±0.4) after activation with seawater ($P<0.05$). No difference was found between the motility of fresh sperm and frozen–thawed sperm with DMSO-sucrose ($P>0.05$) (Fig. 1).

The percentage of live spermatozoa was 60±6%, 43±9% and 19±8% for fresh, frozen–thawed sperm with DMSO-sucrose and glycerol-saline, respectively (Fig. 2). The difference between the fresh and frozen–thawed sperm with glycerol-saline was significant ($P<0.01$). However, no difference was found between the frozen–thawed sperm with DMSO-sucrose and glycerol-saline. A positive correlation was

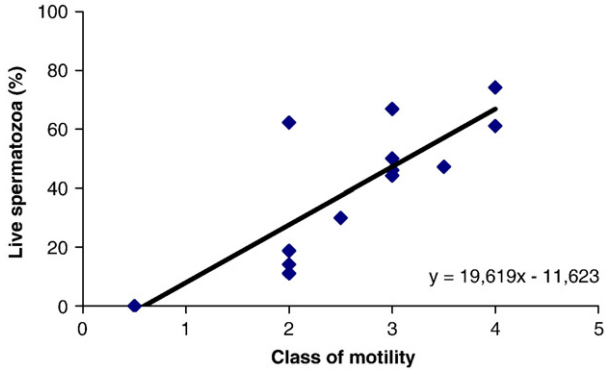

Fig. 3. Correlation between sperm motility and viability determined by differential fluorescent staining for flounder *Paralichthys orbignyanus* sperm ($n=13$ males, Adjusted $R^2=0.61$, $P<0.001$).

Table 2
Floating eggs, fertilization and hatching rates and larvae viability obtained with fresh and cryopreserved sperm of Brazilian flounder *Paralichthys orbignyanus*

Parameters	Fresh	DMSO–sucrose	Glycerol–saline
Floating eggs (%)	94±2[a]	94±1[a]	94±1[a]
Fertilization (%)	73±5[a]	78±5[a]	61±6[a]
Hatching (%)	63±4[a]	67±3[a]	53±7[a]
Larval viability (%)	58±5[a]	60±5[a]	45±8[a]

Data ($n=10$) are represented as mean±SEM. Values sharing the same letters in each line indicate they are not significantly different ($P>0.05$).

verified between sperm motility and viability ($n=13$, Adjusted $R^2=0.61$, $P<0.001$) (Fig. 3).

No significant difference ($P>0.05$) was observed regarding floating eggs, fertilization rate, hatching rate and larvae viability comparing fresh and cryopreserved sperm regardless of the cryoprotectant mixture employed (Table 2).

4. Discussion

In this study, it was demonstrated that the cryopreservation protocols used are efficient for cryopreservation of Brazilian flounder sperm. Sperm cryopreserved with both cryoprotectant solutions resulted in viable larvae, however, considering all quality parameters analyzed, solution made of DMSO–sucrose apparently outperformed the one with glycerol–saline.

DMSO and glycerol are the most commonly cryoprotectants used for cryopreservation of marine fish sperm (Suquet et al., 2000). In most studies, DMSO provides the best results, probably due to its fast penetration into spermatozoa and its interaction with the phospholipids of the sperm membrane (Suquet et al., 2000). Glycerol can be less efficient, possibly due to its toxic effects, osmotic stress, and reduced speed to penetrate into spermatozoa (Stoss, 1983). In addition, the efficiency of glycerol for sperm cryopreservation may be species-specific (Suquet et al., 2000). Spermatozoa cryopreserved with glycerol were motile soon after thawing, even before they were activated with seawater. Sperm motility of fish is controlled through their sensitivity to osmolality and ion concentration. In most species, sperm motility is induced by hypo and hyper-osmotic pressure in freshwater and marine fishes, respectively (to see review Alavi and Cosson, 2006). Thus, it is likely that the high osmolality of the extender solution used along with glycerol does not prevent osmotic stress, allowing the cells to become motile, before being properly activated. Both cryosolutions were cooled (5 °C) before sperm was added, therefore it is likely that activation of sperm treated with glycerol-saline was masked by the low temperature used.

Motility and viability rates of frozen–thawed sperm with DMSO were two fold superior to those obtained using glycerol. In general terms, post-thaw motility and viability of sperm cryopreserved with glycerol were low, however it was not reflected in low fertilization and hatching rates. There are two possible explanations. First, the sperm to egg ratio used could have been high enough to enable the meeting of viable sperm with ovum, even under reduced cell viability. For turbot, the optimal ratio of sperm to egg was found to be 6000:1 (Suquet et al., 1995), however other species may require a higher rate, the optimal sperm to egg ratio can reach 200,000:1 for wolfish *Anarhichas lupus* (Mokness and Pavlov, 1996). The ideal ratio of sperm to egg for Brazilian flounder is unknown and should be evaluated. Second, high fertilization and hatching rates could have been the result of fertilization capacity of sperm that were only vibrating or even immotile. This capacity has already been demonstrated for salmonids (Scott and Baynes, 1980), Atlantic cod *Gadus morhua* (Trippel and Neilson, 1992) and rosy barb *Barbus conchonius* (Amanze, 1994).

The assessment of sperm viability using the "LIVE/DEAD" fluorescence kit has been employed with success for different fish species (DeGraaf and Berlinsky, 2004; Flajshans et al., 2004; Linhart et al., 2005). In the present work, this kit also worked efficiently to evaluate the percentage of live cells after cryopreservation. Moreover, a positive correlation was found between sperm motility and viability for Brazilian flounder. DeGraaf and Berlinsky (2004) working with Atlantic cod and haddock *Melanogranmus aeglefinus* found a positive correlation between these two parameters. On the other hand, there is no correlation between sperm motility and viability for sperm of European catfish *Silurus glanis* (Linhart et al., 2005). According to these authors, a significant correlation was not observed because live spermatozoa include not only motile cells, but also those immotile due to ATP exhaustion. This could be a common feature to species whose life span of sperm is short, as is the case of European catfish (Linhart and Billard, 1994), but not for those with long lasting sperm as Brazilian flounder (more than 60 min, data not published), Atlantic cod (Trippel and Morgan, 1994), and haddock (Rideout et al., 2004).

There are few studies analyzing the quality of larvae obtained with cryopreserved sperm, including analysis of growth rate (Suquet et al., 1998; Chereguini et al., 2002) and percentage of malformed larvae (Rideout et al., 2003; Miskolczi et al., 2005). In the present work, no difference was found in the ratio of malformed larvae between fresh and cryopreserved sperm, the same was also observed for winter flounder and catfish *Clarias gariepinus* (Rideout et al., 2003; Miskolczi et al., 2005).

The results of the present study demonstrate that the cryopreservation protocols used are efficient, but apparently DMSO–sucrose combination outperforms saline–glycerol combination for sperm of Brazilian flounder. However, the best procedure for cryopreservation of sperm should include the investigation of several parameters, including evaluation of other extenders and optimal cryoprotectant concentration (Suquet et al., 2000; Chao and Liao, 2001). This is the first report on successful cryopreservation of Brazilian flounder sperm, this procedure should improve broodstock management techniques for this species and consequently augment the potential for its culture.

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