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Effects of cooling and freezing on sperm motility of the endangered fish piracanjuba *Brycon orbignyanus* (Characiformes, Characidae)

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Abstract

The effects of extenders and cryoprotectants on sperm motility of the endangered fish piracanjuba (*Brycon orbignyanus*, Characiformes, Characidae) after storage at 4-6ºC and at -196ºC were evaluated. In Experiment 1, 20 extender-cryoprotectant combinations (5 extenders x 3 cryoprotectants, plus 5 solutions containing only extender without cryoprotectant) were tested. The extenders tested were: 154 mM NaCl, 200 mM NaCl, Saad (mM: 200 NaCl, 30 Tris), coconut water, and Kurokura (mM: 128.4 NaCl; 2.7 KCl; 1.4 $CaCl_2$; 2.4 $NaHCO_3$). The cryoprotectants (dimethyl sulphoxide - DMSO, methanol, and methylglycol) were added at 10% of the total volume. One aliquot of semen was kept undiluted and served as a control. Motility was subjectively estimated at 0, 1, and 2 days after cold storage. In Experiment 2, the extender-cryoprotectant combinations that produced motility above 70% on Day 0 (Experiment 1) were selected as freezing media. The amount of egg yolk and semen included in each medium was 5 and 10% of the total volume, respectively. Three 0.5-mL straws for each freezing medium were frozen in nitrogen vapor container (Taylor-Wharton, CP 300) at -170°C and then stored in liquid nitrogen. Straws were thawed in a water bath at 60ºC for 8 seconds. Sperm motility one day after cooling was higher in samples diluted in Saad solution (82%), 200 mM NaCl (67%), and 154 mM NaCl - DMSO (77%). Undiluted samples yielded 53% motility. Higher post-thaw sperm motility (66%) was observed in semen cryopreserved in 154 mM NaCl - egg yolk - methylglycol compared to all the other samples. Piracanjuba semen diluted in Saad solution or 200 mM NaCl and stored at 4-6ºC for one day or frozen in 154 mM NaCl - yolk - methylglycol maintained most of its sperm motility.

Keywords: semen, cooling, freezing, sperm motility, fish.

Introduction

The piracanjuba (*Brycon orbignyanus*) is a teleost fish species native to the Paraná-Paraguay River basin (Godoy, 1975). During the rainy season, the piracanjuba migrates upstream through these rivers in order to spawn from November to January. Over-fishing, changes in river current, urbanization, pollution, and hydroelectric dams are some of the reasons why the number of piracanjuba and other endangered migratory fish species is decreasing. The piracanjuba exhibits fast growth in captivity and has an excellent meat quality, indicating that this species can be produced on a commercial scale and thus preventing it from extinction.

Short-term preservation of semen by cooling at 4-6ºC or long-term preservation by freezing semen in liquid nitrogen (-196ºC) are techniques that facilitate assisted reproduction. Storing batches of diluted or undiluted semen in a refrigerator for a couple of days is such an easy procedure that can be adapted to any fish culture. Semen cryopreservation, however, is a technique that demands more steps, such as right choice of extender-cryoprotectants and optimal freezing-thawing rates. Fish semen cryopreservation of Brazilian species has been the subject of some investigation since 1984 (Cóser *et al*., 1984), but only a few studies have been carried out using refrigerated semen (Marques and Godinho, 2004; Murgas *et al.*, 2004; Maria *et al.*, 2006). However, despite the fact that the number of Brazilian fish species is large, the knowledge concerning semen preservation in some species is scarce or even absent. Some freezing protocols developed for one species can be adapted to another. The extender Kurokura that is used to freeze common carp semen (*Cyprinus carpio*; Kurokura *et al*., 1984) and Ginsburg fish Ringer used to freeze African catfish semen (*Clarias gariepinus*; Viveiros *et al*., 2000) was tested with Brazilian piau-açú semen (*Leporinus macrocephalus*; Moraes, 2004) with success. Dimethyl sulphoxide (DMSO) is the most widely-used cryoprotectant in Brazilian characid fish species. For a freezing apparatus, the nitrogen vapor container, also known as "dry-shipper," has been used with great success with many Brazilian species (Carolsfeld *et al*., 2003; Maria *et al.*, 2006; Oliveira, 2006).

The aim of this study was to test the effects of some extender-cryoprotectant combinations during cooling at 4-6ºC or freezing at -196ºC on sperm motility of piracanjuba. All extenders tested (simple and complex saline, and coconut water) were previously used as semen extenders on other fish species.

Materials and Methods

Piracanjuba broodfish were maintained in earthen ponds at the Fish Culture Unit of Itutinga of the Energetic Company of Minas Gerais (CEMIG) and used

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during the spawning season. Males showing running semen under coelomic wall pressure received a single dose of carp pituitary extract (4 mg/kg of body weight, i.m.) to facilitate semen release. After 5 hours of latency time, the urogenital papilla was carefully dried and soft pressure was applied to the male's abdomen. Semen was allowed to drip through the anal fin and was collected directly into sterile test tubes where volume was measured. Immediately after collection, 5 µl of each semen sample was placed on a microscope slide and observed using a light microscope. Any sperm motility (auto-activation) observed was considered urine or water contamination, and this sample was discharged. In immotile samples, sperm motility was subjectively estimated by adding 25 µl of activation medium (50 mM NaCl; Bedore, 1999) and immediately viewed at 400x magnification, and the percentage of visible motile cells was recorded. Only samples containing at least 80% motile cells were used. During manipulation, semen was kept in crushed ice. Then, it was transported to the laboratory either in a polystyrene box filled with crushed ice (Experiment 1) or in straws frozen in nitrogen vapor (Experiment 2). The mean sperm concentration of all males was determined by counting diluted (1:1000) semen in a Neubauer counting chamber. All experiments were carried out in triplicate.

Experiment 1. Extender-cryoprotectant combinations during the cooling of semen

Semen of each male was diluted 1:5 (semen:total volume) in 20 extender-cryoprotectant combinations (5 extenders x 3 cryoprotectants, plus 5 solutions containing only extender without a cryoprotectant). The extenders tested were based on our previous work (Maria *et al*., 2006) and were as follows: 1) 154 mM NaCl (physiological saline solution, 0.9%; 285 mOsm); 2) 200 mM NaCl (404 mOsm); 3) Immobilizing Saad solution (200 mM NaCl, 30 mM Tris; 429 mOsm); 4) Coconut water (Kero-coco™; 0.02% calcium; 0.02% sodium; 0.01% magnesium; 0.32% potassium; 0.01% phosphorus; 5% carbohydrates; 321 mOsm); and 5) Kurokura (128.4 mM NaCl; 2.7 mM KCl; 1.4 mM $CaCl_2$; 2.4 mM $NaHCO_3$; 240 mOsm). Extender osmolalities were measured using an advanced micro-osmometer (model 3MO, Needham Hieghts, Massachussets) at the Wageningen Institute of Animal Sciences in The Netherlands.

Dimethyl sulphoxide (DMSO), methanol, and methylglycol were tested as cryoprotectants and were added to the extender at 10%. One aliquot of semen was kept undiluted and served as a control. All semen samples were kept in 5-mL open tubes at 4-6ºC, and sperm motility was evaluated after 0, 1, and 2 days of storage.

Experiment 2. Freezing medium during semen cryopreservation

The extender-cryoprotectant combinations that produced sperm motility above 70% on Day 0 of cooling (Experiment 1) were selected as freezing media. Egg yolk at 5% (Maria *et al*., 2006) and semen (1:10 total volume) were added to each medium. The following 10 cryoprotectant-extender combinations were tested: DMSO combined with 154 mM NaCl, Saad, 200 mM NaCl, coconut water, or Kurokura; methylglycol combined with 154 mM NaCl, Saad, or 200 mM NaCl; or methanol combined with 154 mM NaCl or Saad.

Three 0.5-mL straws were filled with semen diluted in each of the selected 10 combinations, placed in nitrogen vapor container (Taylor-Wharton, CP 300, "dry shipper") at -170ºC for 24 h, and then plunged into liquid nitrogen for storage. Sperm motility was evaluated after thawing at 60ºC in water bath for 8 seconds (Maria *et al.*, 2006).

Statistical analysis

All data on motility are expressed as mean ± standard deviation (SD). All statistical analyses were carried out using Sisvar package (Sisvar; Ferreira, 1999). Percentage motile sperm cells of fresh and cryopreserved semen for each experiment were analyzed using ANOVA and followed by Scott-Knott test. The residuals from different models were tested for normal distribution. When normal distribution was not found, arcsine transformation was used. Data were considered to be significant at $P < 0.05$.

Results

Semen volume and sperm motility

Body weight and sperm concentration of males used in these experiments are depicted in Table 1. Sperm motility varied from 95 to 100% in all samples. Semen volume was always above 10 ml.

Extender osmolality

Some spontaneous motility (10-20%) was observed in semen diluted in Kurokura solution and the osmolality observed in this extender was the lowest one (240 mOsm) compared to the other extenders. Semen diluted in all the other extenders remained immotile until activation with 50 mM NaCl.

Table 1. Body weight and sperm concentration of piracanjuba broodfish.

Male	Body weight (kg)	Sperm concentration (x 10^9/ml)
1	1.2	4.2
2	0.8	9.2
3	0.9	6.9
4	1.0	2.8
5	1.1	4.6
6	0.9	3.4
Mean (± SD)	0.98 ± 0.15	5.2 ± 2.4

Experiment 1. Extender-cryoprotectant combinations during the cooling of semen

Motilities above 95% were observed in semen samples diluted 1:5 (semen:total volume) in all five extenders without cryoprotectant and undiluted control semen on Day 0 (Table 2). When cryoprotectants were added to the extenders, motility decreased to below 70% in the following combinations: 200 mM NaCl - methanol, coconut water - methanol, coconut water - methylglycol, Kurokura - methanol, and Kurokura - methylglycol.

Higher ($P<0.05$) motility rates were observed on semen diluted in 154 mM NaCl - DMSO, 200 mM NaCl only, and Saad only, compared to undiluted control semen or semen diluted in the other solutions, on Day 1 after cooling. Sperm motility was very low (30% or below) in all samples, on Day 2 after cooling.

Table 2. Motility (%; mean ± SD; n = 3 fish) of piracanjuba semen diluted 1:5 (semen:total volume) in different combinations of extenders and cryoprotectants (10%) and cooled at 4-6°C for 2 days (Experiment 1).

Extenders	Cryoprotectants	Cooling storage (days)		
		0	1	2
154 mM NaCl	DMSO	97 ± 3 [a]	77 ± 6 [a]	30 ± 20 [a]
	Methanol	98 ± 2 [a]	37 ± 11 [c]	0 [b]
	Methylglycol	98 ± 2 [a]	43 ± 11 [c]	23 ± 15 [a]
	--	100 ± 0 [a]	60±10 [b]	0 [b]
200 mM NaCl	DMSO	88 ± 3 [a]	27 ± 15 [d]	0 [b]
	Methanol	57 ± 15 [d]	0 [e]	0 [b]
	Methylglycol	90 ± 5 [a]	20 ± 10 [d]	0 [b]
	--	100 ± 0 [a]	67 ± 6 [a]	17 ± 15 [a]
Saad[1]	DMSO	83 ± 6 [b]	17 ± 15 [d]	0 [b]
	Methanol	85 ± 13 [b]	27 ± 15 [d]	13 ± 6 [a]
	Methylglycol	95 ± 5 [a]	13 ± 13 [d]	10 ± 10 [a]
	--	100 ± 0 [a]	82 ± 3 [a]	17 ± 11 [a]
Coconut water[2]	DMSO	82 ± 10 [b]	0 [e]	0 [b]
	Methanol	0 [f]	0 [e]	0 [b]
	Methylglycol	40 ± 10 [e]	7 ± 6 [e]	0 [b]
	--	95 ± 0 [a]	0 [e]	0 [b]
Kurokura[3]	DMSO	73 ± 6 [c]	57 ± 6 [b]	10 ± 10 [a]
	Methanol	65 ± 9 [c]	50 ± 10 [b]	0 [b]
	Methylglycol	68 ± 3 [c]	27 ± 21 [d]	0 [b]
	--	100 ± 0 [a]	45 ± 9 [c]	0 [b]
Undiluted semen		100 ± 0 [a]	53 ± 6 [b]	17 ± 6 [a]

[1]Saad (mM): NaCl 200; Tris 30.

[2]Coconut water (%): 0.02 calcium; 0.02 sodium; 0.01 magnesium; 0.32 potassium; 0.01 phosphorus; 5 carbohydrates.

[3]Kurokura (mM): NaCl 128.4; KCl 2.7; $CaCl_2$ 1.4; $NaHCO_3$ 2.4.

[a-d] Means followed by different superscripts in a column differ ($P < 0.05$).

Experiment 2. Freezing medium during semen cryopreservation

Straws placed inside the nitrogen vapor vessel reached -170°C after 329 seconds, and a freezing rate of approximately -35.6 °C/min^{-1} between +21 and –170 °C was observed. The highest post-thaw motility was observed when semen was cryopreserved in 154 mM NaCl - egg yolk - methylglycol compared to semen cryopreserved in all the other freezing media (Table 3).

Table 3. Motility (%; mean ± SD; n = 3 fish) of cryopreserved piracanjuba semen diluted 1:10(semen:total volume) in different extenders, 10% cryoprotectants, and 5% egg yolk (Experiment 2).

Freezing media		Motility (%)
Extenders	Cryoprotectant (10%)	
154 mM NaCl	DMSO	8 ± 2 [e]
	Methanol	7 ± 4 [e]
	Methylglycol	66 ± 9 [a]
200 mM NaCl	DMSO	16 ± 5 [d]
	Methylglycol	44 ± 5 [b]
Saad[1]	DMSO	33 ± 7 [c]
	Methanol	4 ± 4 [e]
	Methylglycol	26 ± 5 [c]
Coconut water[2]	DMSO	10 ± 8 [e]
Kurokura[3]	DMSO	5 ± 4 [e]

[1]Saad (mM): NaCl 200; Tris 30.

[2]Coconut water (%):0.02 calcium; 0.02 sodium; 0.01 magnesium; 0.32 potassium; 0.01 phosphorus; 5 carbohydrates.

[3]Kurokura (mM): NaCl 128.4; KCl 2.7; $CaCl_2$ 1.4; $NaHCO_3$ 2.4.

[a-e] Means followed by different superscripts differ ($P < 0.05$).

Discussion

Sperm concentration of Brazilian fish species varies from 4.3 x 10^9 to 66.5 x 10^9 sperm cells per ml (Bedore, 1999). In this study, sperm concentration was 5.2 x 10^9 ± 2.4 x 10^9 cells per ml. Piracanjuba sperm concentration has previously been reported as 8.2 x 10^9 ± 2.3 x 109 (Murgas *et al*., 2004) and 10.0 x 10^9 ± 4.3 x 10^9 sperm cells per ml (Bedore, 1999).

Fish spermatozoa are immotile in seminal plasma. When semen is released in aquatic environment, osmolality goes down (in freshwater species), and sperm motility is initiated. All extenders used in this study were able to keep sperm immotile after dilution (Day 0). The only exception was Kurokura solution that activated 10-20% of sperm. Kurokura solution possessed the lowest osmolality (240 mOsm) compared to the other extenders tested. It is possible that such a low osmolality may have partially activated the piracanjuba sperm cells. Kurokura is a semen extender formerly used for fish species cultivated in Europe, such as common carp (*Cyprinus carpio*; Kurokura *et al*., 1984) and tench (*Tinca tinca*; Rodina *et al*., 2004). In tench fish, various concentrations of urine usually contaminate semen that cause decreased sperm motility and thus result in a poor fertilization rate. Without special care, tench sperm cannot be stored even for a short time without using an immobilizing solution such as Kurokura solution. In piracanjuba, it is possible that Kurokura would inhibit spontaneous sperm motility at higher osmolalities such as 343 mOsm (Rondina *et al*., 2004).

The use of extenders may stabilize physical-chemical conditions during storage and thereby prolong the life of spermatozoa in storage (Stoss, 1983). Semen diluted in coconut water or in Kurokura combined with all cryoprotectants showed a significant ($P<0.05$) and immediate (Day 0) reduction on sperm motility compared to semen diluted in the same extenders without a cryoprotectant and in undiluted control semen. A very strong and negative effect on sperm motility was observed when coconut water was combined with methanol. No motility was observed immediately after adding semen to the coconut water-methanol solution. Similarly, a negative effect of the coconut-water-methanol was observed in piracanjuba and pacu (*Piaractus mesopotamicus*) semen (Bedore, 1999). Thus, the coconut-water-methanol combination is not recommended as a solution to be used during storage of piracanjuba semen.

One day after cooling, the highest motilities were observed when piracanjuba semen was diluted in

154 mM NaCl - DMSO, 200 mM NaCl, and Saad compared to undiluted control semen and all the other extender-cryoprotectant combinations. There was no improvement on sperm motility when cryoprotectants were added to the extenders, except for the 154 mM NaCl - DMSO combination.

Motilities varying from 0 to 30% were observed on Day 2 after cooling in all piracanjuba semen samples, diluted or not. An increase on bacterial growth has been observed when piracanjuba semen was collected using the same method described in the current experiment (Isaú, 2006). Bacterial growth may alter physical-chemical characteristics of semen and compete for oxygen. However, when semen was collected directly from the urogenital papilla without any contact to fish skin and diluted at a higher ratio of 1:10, motility above 37% was observed after 7 days at 4-6°C (Maria *et al.,* 2006).

The highest post-thaw motility was observed when semen was cryopreserved in 154 mM NaCl as extender and methylglycol as cryoprotectant, compared to samples cryopreserved in DMSO or methanol (Experiment 2). Similar results were observed for semen of the same species when 154 mM NaCl was combined with the same cryoprotectant (Maria *et al.*, 2006). When 200 mM NaCl was used as extender, samples cryopreserved in methylglycol also produced a higher motility rate than samples cryopreserved in DMSO. Of all combinations tested, 154 mM NaCl - methylglycol produced the highest post-thaw motility. This result suggests that methylglycol has the highest capacity of preserving piracanjuba spermatozoon motility during the events of freezing and thawing. Methylglycol, the monoethyleneglycol methyl ether, was firstly tested as cryoprotectant agent for fish semen in another study of this research group (Maria *et al.*, 2006). Semen of piracanjuba was cryopreserved in methylglycol, DMSO, or methanol as cryoprotectants and combined with 154 mM NaCl, BTS™, or M III™ as extenders. BTS™ and M III™ are commercial glucose-based extenders for boar semen (Minitub™) that have been producing good results in fish semen cryopreservation. Highest motilities were observed when semen was cryopreserved in methylglycol compared to DMSO or methanol despite the extender used.

Sodium chloride based solutions from 100 to 200 mM have low risk of hypo- or hyperosmotic damage to salmonid sperm (Scott and Baynes, 1980). The use of simple solutions as semen extender produced as high as and sometimes even higher motility rate than more complex solutions such as coconut water. However, when semen was cryopreserved in Saad-methylglycol, post-thaw motility was lower than samples cryopreserved in 154 mM NaCl or 200 mM NaCl combined with the same cryoprotectant. Saad solution has a higher osmolality (429 mOsm) than 200 mM NaCl (404 mOsm) or 154 mM NaCl (285 mOsm). It is possible that this higher osmolality caused some hyperosmotic damage to cells during the cryopreservation process. In contrast, African catfish semen could be successfully cryopreserved in Ginzburg fish Ringer (a saline extender) at 244 mOsm but not at 220 mOsm. In a 220-mOsm extender, sperm motility was activated before freezing (Viveiros, 2005).

Although fertility was not evaluated in this study, it has been demonstrated that post-thaw motility is highly correlated with the capacity of piracanjuba sperm to fertilize eggs. A post-thaw motility of 60% yielded 58% hatching when piracanjuba semen was cryopreserved in NaCl-methylglycol-yolk medium (Maria *et al*, 2006).

Based on this study, we concluded that piracanjuba semen can be stored for longer than one day when diluted in 200 mM NaCl or in Saad solution. Cryoprotectants are not needed during piracanjuba semen preservation at 4-6°C. Semen can be successfully cryopreserved in a freezing medium containing 154 mM NaCl, 10% methylglycol, and 5% egg yolk.

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