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Use of glucose or BTS™ combined with DMSO or methylglycol under two different freezing protocols for the cryopreservation of sperm from the common curimatã (*Prochilodus brevis*)

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Abstract

This study evaluated the effect of glucose or Beltsville Thawing Solution (BTS™) combined with dimethyl sulfoxide (DMSO) or methylglycol (MG) under two different freezing protocols on the kinetics and morphology of cryopreserved *Prochilodus brevis* sperm. The semen samples were diluted using one of four different treatments (glucose+DMSO, glucose+MG, BTS™+DMSO, and BTS™+MG), loaded into 0.25-ml straws and subjected to two different freezing processes (programmed freezing machine and dry shipper). After 10 days, the semen samples were thawed, and the sperm morphology and kinetics were evaluated. The physicochemical parameters of the semen *in natura* were similar to those observed in other studies of Characiformes, indicating the feasibility of semen cryopreservation. Glucose, when used as a diluent with the cryoprotectant MG (glucose+MG), yielded higher percentages of mobile spermatozoa after freezing in a dry shipper (76.88 ± 4.84%) and in a programmed freezing machine (70.95 ± 1.76%) compared with the combination of glucose and DMSO. Moreover, the glucose+MG treatment yielded a higher sperm velocity (curvilinear velocity: 79.52 ± 2.88 μm s^{-1}; straight-line velocity: 45.46 ± 3.01 μm s^{-1}; average path velocity: 67.92 ± 3.08 μm s^{-1}) than the other studied treatments, and a higher amount of normal sperm (74.56 ± 0.77%) was observed in the semen samples cryopreserved using a programmed freezing machine. The sperm abnormalities observed included a bent tail morphology. Therefore, the use of glucose+MG in combination with either a dry shipper or a programmed freezing machine is recommended for the cryopreservation of *P. brevis* sperm because these methods yielded high numbers of motile and morphologically normal spermatozoa.

Keywords: fish; reproduction; seminal cryopreservation.

Introduction

Prochilodus brevis (Steindachner, 1875), popularly known as the common curimatã, is a Characiformes fish distributed in the inland and coastal watersheds of the Northeast Region of Brazil (Chellappa *et al*., 2009). Despite the considerable economic value, approximately 2000 tons per year according to the National Department of Works Against the Droughts of Brazil, and ecological importance of *P. brevis* in this region (Araújo and Gurgel, 2002), the reproductive biology of this species is poorly understood. Moreover, this species is threatened by the overfishing of mature individuals and the construction of dams, which impair migration toward breeding habitats (Nascimento *et al*., 2012).

Therefore, researchers have shown increasing interest in the study of assisted reproduction for this species. One area of particular interest is the cryopreservation of semen for use in aquaculture production and the preservation of genetic material in conservation programs (Carolsfeld *et al*., 2003). Several studies have shown that cryopreservation exhibits strong potential for improving fish breeding. Cryopreservation presents several benefits, including the synchronization of gamete availability, easier transportation, the prevention of gamete aging, the conservation of genetic variability, reducing the maintenance costs of breeding stock, and the potential for the exchange of genetic material among laboratories (Carneiro *et al*., 2006).

However, to achieve good sperm quality after thawing, various factors, such as a good freezing solution and appropriate cooling rates with appropriate equipment, must be considered. Hence, specific parameters, such as motility and morphology, need to be examined (Felizardo *et al*., 2010). In addition, the semen-and-extender interaction is species-specific: a particular extender may prove effective for freezing the semen of one species but yield poorer post-thaw quality with the semen of other species (Salmito-Vanderley *et al*., 2014).

Few studies have attempted to characterize and cryopreserve *P. brevis* sperm, and their results show low motility after thawing. In addition, few studies have examined the use of a programmed freezing machine for the cryopreservation of sperm from characiform fish (Salmito-Vanderley *et al*., 2014).

This study evaluated the effect of glucose or Beltsville Thawing Solution (BTS™) combined with dimethyl sulfoxide (DMSO) or methylglycol (MG) under two different freezing protocols on the kinetics and morphology of cryopreserved *P. brevis* sperm.

The use of glucose in combination with MG and a programmed freezing machine for the cryopreservation of *P. brevis* semen was found to yield higher proportions of motile and normal spermatozoa after thawing than the other approaches analyzed.

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Materials and Methods

Animals

Eight *P. brevis* males (average age, three years) from the breeding stock of the Biotechnology of Fish Reproduction Laboratory (3°43'47"S and 38°30'37"W) at the Integrated Center for Biotechnology of the State University of Ceará were studied. The animals were given two daily meals containing 28% crude protein to equal a daily feed amount of 3% of their body weight. Animals exhibiting (n = 8) a hyperemic urogenital papilla and that released semen under a light abdominal massage were selected, weighed, and measured. The project was approved by the Ethics Committee for Animal Use at the State University of Ceará (Case Number: 12776936-6).

Semen collection, processing, and freezing

i. Breeding males were captured from tanks (~28ºC) and hormonally induced with a single dose of carp pituitary extract (CPE: 3 mg kg^{-1}) at the base of the pectoral fin using the intracoelomic route. Eighteen hours after induction, semen was collected as follows: The animals were individually captured and sedated through the application of a solution of clove oil (Eugenol; Sigma-Aldrich Ltd., MO, USA; 1 ml of clove oil:10 ml of absolute alcohol:10 L of tank water) until the animals visibly lost balance, as demonstrated by the fish belly facing upward. The urogenital papilla was then dried using a paper towel, and an abdominal massage was performed in the cranial to caudal direction, avoiding contamination of the semen with water, blood, feces, or urine. Semen was collected in graduated polyethylene tubes, which were labeled and stored in a thermally insulated polystyrene box (~10ºC). Contaminated samples and samples that showed sperm with less than 80% objective motility when activated with tank water were not used. The following characteristics of the valid samples obtained from each animal were assessed:

ii. Osmolality: An aliquot of semen (100 µl) was obtained from each animal, and the seminal osmolality was measured using a Peltier cooling digital osmometer (Roebling, Germany).

iii. pH: Semen (10 µl) from each animal was tested using pH test strips (Merck, Germany).

iv. Volume: The volume was measured using graduated polyethylene tubes.

v. Sperm morphology: A 5-µl sample of sperm was fixed in 50 µl of formalin citrate solution, and 10 µl of the fixed sperm was then mixed with 3 µl of Rose Bengal dye (Vetec Fine Chemicals Ltd., Rio de Janeiro, Brazil). Four microliters of this mixture were smeared on glass slides. This procedure was performed twice (using clean blades each time), and 100 spermatozoa on each slide were examined. For each male, a total of 200 sperm were analyzed and cryopreserved. The data obtained from the fresh and post-thawed sperm were then compared. The damaged observed in the spermatozoa were classified according to Galo *et al*. (2011).

vi. Sperm concentration: Semen samples from each animal were fixed in 4% formalin citrate solution (1 µl of semen: 4 ml of fixative; 1:4,000). Twenty microliters from the fixed sample were deposited in a Neubauer chamber and observed under an optical microscope (400X).

vii. Cryopreservation: The extenders used for cryopreservation were BTS™ (Minitub, 318 mOsm) and commercial glucose (Fresenius Kabi Brazil Ltd., 252 mOsm). The cryoprotectants DMSO (Vetec Fine Chemicals Ltd., Rio de Janeiro, Brazil) and MG (Vetec Fine Chemicals Ltd., Rio de Janeiro, Brazil) were used at 10% dilution (v/v).

viii. BTS™ (composed of 79.90 g of glucose, 12.71 g of sodium citrate, 2.65 g of EDTA, 2.65 g of sodium bicarbonate, 1.59 g of potassium chloride and 0.50 g of gentamicin sulfate in 1.00 L of distilled water) was prepared by diluting 50 g of BTS™ in 100 ml of distilled water as recommended by the manufacturer.

The following treatments were applied: (T1) glucose+10% DMSO, (T2) glucose+10% MG, (T3) BTS™+10% DMSO and (T4) BTS™+10% MG. The total semen of each male (n = 8 males) was diluted (1:6 semen:extender) in all of the treatments and loaded in 0.25-ml straws (n = 6 replicate straws per treatment and per male), identified, sealed at the ends with polyvinyl alcohol, and maintained at 10ºC for 10 min. Simultaneously, three straws from each treatment were placed into a programmed freezing machine (Dominium K, BIOCOM™, Brazil), and three other straws were placed on the lower part of a rack and placed inside a Model CP300 dry shipper (Taylor-Wharton). In total, 192 straws (three replicate straws x four media x two freezing methods x eight males) were cryopreserved.

For cryopreservation using the programmed freezing machine, a two-step freezing protocol was used. First, the milt was cooled from 10 to -12°C at a rate of -3°C per minute, crystallized for 1 min, cooled again from -12 to -60°C at a rate of -3°C per minute and stabilized for 30 min.

The straws were maintained in the dry shipper for 15 min or in the programmed freezing machine for approximately 55 min, transferred to liquid nitrogen and stored for 10 days at -196°C.

Defrosting and evaluation of sperm kinetics

The samples were thawed by immersion in a thermal bath at 25°C for 30 s. After thawing, the semen was activated in a NaCl solution (100 mOsm), and the sperm kinetics were observed in a Makler™ counting

chamber placed in a phase-contrast microscope (Nikon H550S™, ECLIPSE 50i, Japan) equipped with a green filter and using the pH1 position at 400X magnification. The microscope was connected to a video camera (Basler Vision Technologies™ A312FC, Ahrensburg, Germany), and video was recorded at a frame rate of 25 images/s. Each image (n = 25) was analyzed using the standard settings for fish included with Sperm Class Analyzer™ software (SCA version 5.0, Microptics SL, Barcelona, Spain). At least 3,000 sperm were analyzed per straw (n = 3 straws), and the total motility, curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP) were measured.

Statistical analysis

The study followed a completely randomized design with a 2 x 4 (freezing methods x treatments) factorial arrangement. After a normality test, the semen parameter data were subjected to analyses of variance to test the effects of the freezing method, the treatment protocol (dilution), and the interaction between these parameters. The significance of these effects was tested by comparing the means using Tukey's test ($P < 0.05$). The numbers of sperm exhibiting broken, twisted, or corrugated tails were compared using the Kruskal-Wallis test ($P < 0.05$). A study of simple Pearson correlations was performed to assess the degree of association between the variables measured in this study. The data were analyzed using SAS statistical software version 8.0 (2000).

Results

Characterization

The fish had an average length of 30.71 ± 0.76 cm (mean ± S.D.) and a mean weight of 524.29 ± 47.91 g (mean ± S.D.). The mean values of the semen parameters observed *in natura* are presented in Table 1.

Table 1. Means ± standard deviations of *P. brevis* semen parameters *in natura* (n = 8 males).

Parameter	Mean ± S.D.
pH	8.21 ± 0.27
Volume (ml)	1.24 ± 0.33
Osmolarity (mOsm)	250.29 ± 25.84
Concentration (x 10^9 sptz ml^{-1})	15.12 ± 2.77
Total motility (%)	98.23 ± 1.64
VCL (μm s^{-1})	126.60 ± 10.34
VSL (μm s^{-1})	65.37 ± 10.66
VAP (μm s^{-1})	110.76 ± 15.18

VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity.

Kinetic and morphological analysis of semen

The choice of freezing method had no effect on the kinetic parameters evaluated, but the treatment did exert an effect (Table 2). T2 (glucose+MG) resulted in a significantly higher ($P < 0.05$) VCL compared with the other treatments. T1 (glucose+DMSO) and T3 (BTS™+DMSO) yielded significantly lower VCL values than the other treatments ($P < 0.05$). The same pattern was observed for VSL and VAP with the exception that T1 resulted in significantly lower values for the VSL and VAP than T3.

Table 2. Means and standard errors of the kinetic parameters (curvilinear velocity - VCL, straight-line velocity - VSL, and average path velocity - VAP) of *P. brevis* semen frozen using various treatments (n = 6 replicate straws x 8 males for each treatment) and then thawed.

Treatment	Kinetic parameters		
	VCL (μm s^{-1})	VSL (μm s^{-1})	VAP (μm s^{-1})
T1	39.03 ± 0.92 ^{c}	17.39 ± 1.08 ^{d}	28.33 ± 1.08 ^{d}
T2	79.52 ± 2.88 ^{a}	45.46 ± 3.01 ^{a}	67.92 ± 3.08 ^{a}
T3	44.07 ± 1.05 ^{c}	28.74 ± 1.07 ^{c}	38.60 ± 1.15 ^{c}
T4	63.68 ± 2.71 ^{b}	36.85 ± 2.04 ^{b}	54.06 ± 2.08 ^{b}

Different letters in the same column indicate results that were found to be significantly different according to Tukey's test ($P < 0.05$). T1: glucose+DMSO; T2: glucose+MG; T3: BTS™+DMSO; T4: BTS™+MG.

The total motility and percentages of normal and bent-tailed sperm in the post-thawed semen were affected not only by the treatment used but also by the freezing method employed (Table 3). Significant differences ($P < 0.05$) in the total motility (%) between the freezing methods were only observed with T4: the

semen cryopreserved using the programmed freezing machine exhibited a higher total motility (50.86 ± 3.36%) than the semen frozen using the dry shipper (37.38 ± 5.06%). T2 resulted in the highest percentage of mobile spermatozoa among the treatments, regardless of the freezing method employed. However, the use of the programmed freezing machine (PFM) yielded a lower coefficient of variation (CV) than the dry shipper (DS; PFM CV = 7.01; DS CV = 17.8).

The analysis of damaged spermatozoa obtained after cryopreservation using the dry shipper revealed that T4 yielded the highest percentage of normal sperm among the treatments (79.50 ± 2.27%). In contrast, using the programmed freezing machine, T2 (74.56 ± 0.77%) presented the highest percentage of normal sperm.

For all of the analyzed treatments, a greater proportion of bent-tail pathology was obtained through cryopreservation using the programmed freezing machine. Using this freezing method, T3 (44.81 ± 1.96%) presented the highest percentage of cells with bent tails, and T2 (23.25 ± 0.65%) presented the lowest percentage. The analysis of cryopreservation using the dry shipper showed that the highest percentage of cells with bent-tail pathology were obtained using T1 (37.38 ± 2.00%). T2 (23.88 ± 2.96%), T3 (25.19 ± 3.94%), and T4 (19.87 ± 2.38%) yielded lower percentages of cells with this pathology, and no significant differences ($P > 0.05$) were observed among these treatments.

Table 3. Means ± standard errors of the variables recorded as percentages of mobile spermatozoa. The results were obtained for *P. brevis* semen after thawing as a function of the interaction of the treatment and freezing method (n = 3 replicate straws x 8 males from each treatment and freezing method).

Variable	Treatment				
	Method	T1	T2	T3	T4
MOT (%)	DS	23.64 ± 2.26^{Ca}	76.88 ± 4.84^{Aa}	41.12 ± 3.14^{Ba}	37.38 ± 5.06^{Bb}
	PFM	18.10 ± 2.87^{Da}	70.95 ± 1.76^{Aa}	37.58 ± 2.62^{Ca}	50.86 ± 3.36^{Ba}
NORMAL (%)	DS	53.19 ± 1.55^{Cb}	72.88 ± 4.18^{Ba}	72.19 ± 3.89^{Ba}	79.50 ± 2.27^{Aa}
	PFM	62.38 ± 2.10^{Ba}	74.56 ± 0.77^{Aa}	53.31 ± 2.07^{Cb}	63.75 ± 1.75^{Bb}
BENT TAIL (%)	DS	37.38 ± 2.00^{Aa}	23.88 ± 2.96^{Ba}	25.19 ± 3.94^{Bb}	19.87 ± 2.38^{Bb}
	PFM	33.13 ± 1.89^{Ba}	23.25 ± 0.65^{Ca}	44.81 ± 1.96^{Aa}	34.81 ± 1.61^{Ba}

Different uppercase letters in the same row indicate differences between treatments, whereas different lowercase letters in the same column indicate differences between freezing methods. The significance of these differences were evaluated using PDIFF ($P < 0.05$). T1: glucose+DMSO; T2: glucose+MG; T3: BTS™+DMSO; T4: BTS™+MG; DS: dry shipper; PFM: programmed freezing machine.

The choice of freezing method did not affect the appearance of tail defects (broken tail, coiled tail and corrugated tail) in the sperm after thawing; however, the choice of treatment did affect tail defects (Table 4). Among the treatments, T1 conferred a greater number of pathological defects, whereas T3 and T4 yielded the lowest number of abnormalities. Among the abnormalities recorded in Table 4, broken-tail pathology was the most frequent. None of the following sperm abnormalities were observed: free head, macrocephaly, microcephaly, distal and proximal cytoplasmic droplets, and degenerated flagella.

Table 5 presents the correlations found between the kinetic, morphological, and physicochemical characteristics of *P. brevis* semen. The total motility was positively correlated with sperm velocities, including VCL (0.82523, $P < 0.001$), VSL (0.76214, $P < 0.001$), and VAP (0.82256, $P < 0.001$). In addition, a positive correlation was observed between total motility and normal sperm morphology (0.45908, $P < 0.05$), and a negative correlation was observed between total motility and the bent-tail (-0.40088, $P < 0.05$), broken-tail (-0.28728, $P < 0.05$), and corrugated-tail (-0.42494, $P < 0.05$) morphologies.

Correlations were observed among the physicochemical characteristics: a positive correlation was observed between seminal pH and seminal osmolality (0.41043, $P < 0.05$), and negative correlations were observed between pH and volume (-0.37097, $P < 0.05$) and between pH and sperm concentration (-0.79237, $P < 0.001$). In addition, the seminal volume was positively correlated with a coiled-tail morphology (0.25476, $P < 0.05$) and the sperm concentration (0.67561, $P < 0.001$); however, the sperm concentration was negatively correlated with seminal osmolality (-0.27071, $P < 0.05$).

Table 4 Percentages of defects in the tail of post-thaw sperm (broken tail, coiled tail, and corrugated tail) of semen from *P. brevis* subjected to various treatments (n = 6 replicate straws x 8 males from each treatment).

Treatment	Defects (%)					
	Broken		Coiled		Corrugated	
	Mean ± S.D	Min ≤ Median ≤ Max	Mean ± S.D	Min ≤ Median ≤ Max	Mean ± S.D	Min ≤ Median ≤ Max
T1	4.12 ± 2.87 [a]	0 ≤ 4 ≤ 9.5	0.37 ± 0.56 [a]	0 ≤ 0 ≤ 2	2.46 ± 1.70[a]	0 ≤ 2 ≤ 5
T2	1.75 ± 1.72 [b]	0 ≤ 1.5 ≤ 6.5	0.53 ±0.96 [a]	0 ≤ 0 ≤ 3.5	0.43 ± 0.57 [b]	0 ≤ 0.25 ≤ 2
T3	1.81 ± 1.55 [b]	0 ≤ 1.25 ≤ 6	0.37 ± 0.43[a]	0 ≤ 0.5 ≤ 1.5	0.06 ± 0.17 [c]	0 ≤ 0 ≤ 0.5
T4	0.84 ± 0.81[b]	0 ≤ 0.75 ≤ 2.5	0.12 ± 0.22[a]	0 ≤ 0 ≤ 0.5	0.06 ± 0.17[c]	0 ≤ 0 ≤ 0.5

Different letters in the same column indicate results that were found to differ significantly based on the Kruskal-Wallis test ($P < 0.05$). T1: glucose+DMSO; T2: glucose+MG; T3: BTS™+DMSO; T4: BTS™+MG. The values represent the means ± standard deviations (Mean ± S.D) and medians (Median), minimum (Min) and maximum (Max) values (Min ≤ Median ≤ Max).

Table 5 Correlation matrix among the kinetic, morphological, and physicochemical characteristics of *P. brevis* semen.

	Total Motility	VCL	VSL	VAP	Normal	Bent tail	Broken tail	Corrugated tail	Coiled tail	pH	Volume	Osmolarity
VCL	0.82523**	-	-	-	-	-	-	-	-	-	-	-
VSL	0.76214**	0.90721**	-	-	-	-	-	-	-	-	-	-
VAP	0.82256**	0.98020**	0.96734**	-	-	-	-	-	-	-	-	-
Normal	0.45908*	0.54447**	0.47695**	0.52504**	-	-	-	-	-	-		
Bent tail	-0.40088*	-0.49589**	-0.40962**	-0.46819**	-0.96063**	-	-	-	-	-	-	-
Broken tail	-0.28728*	-0.33259*	-0.32468*	-0.32951*	-0.40869*	0.16607	-	-	-	-	-	-
Corrugated tail	-0.42494*	-0.35063*	-0.44418*	-0.40396*	-0.43729*	0.24648*	0.46994**	-	-	-	-	-
Coiled tail	0.01648	-0.00385	0.00286	0.00491	-0.35806*	0.25014*	0.29223*	0.23702	-	-	-	-
pH	-0.11316	-0.08651	-0.08417	-0.10136	-0.00070	-0.02362	0.04140	0.10452	0.03006	-	-	-
Volume	-0.02557	-0.07909	-0.14484	-0.11191	-0.05925	0.08050	-0.12800	-0.00942	0.25476*	-0.37097*	-	-
Osmolarity	-0.09445	-0.10339	-0.17703	-0.13505	0.08524	-0.08285	-0.12598	0.07968	0.05871	0.41043*	-0.02261	-
Concentration	0.10418	0.03441	0.02249	0.03703	0.02401	-0.00879	-0.03629	-0.07729	-0.00538	-0.79237**	0.67561**	-0.27071*

(*) significant at $P < 0.05$; (**) significant at $P < 0.001$

Discussion

The present study analyzed the physicochemical parameters of *P. brevis* semen *in natura* by evaluating the effects of different media and freezing equipment on the kinetics and morphology of *P. brevis* semen. The evaluation of post-thaw sperm kinetics was conducted using the Computer-Assisted Semen Analysis (CASA) system.

Knowledge of semen characteristics is important for evaluating the quality of semen that will be used in artificial breeding and laboratory experiments (Orfão *et al*., 2011). This knowledge becomes even more important when evaluating the effects of cryopreservation. Successful cryopreservation is related to semen quality because some loss of semen quality is expected after cryopreservation.

Among the measured characteristics, pH is particularly important because it is related to the regulation of sperm motility in fish (Cosson, 2004). In salmonids, this factor may be responsible for the acquisition of motility during the passage of sperm from the testicle to the spermatic duct (Morisawa and Morisawa, 1986, 1988; Billard *et al*., 1995). Thus, knowledge of the pH is fundamentally important to the development of an appropriate freezing solution for sperm cryopreservation (Maria *et al*., 2010). According to Tabares *et al*. (2005), the sperm pH varies from 6.5 to 8.5, and the pH of *P. brevis* semen found in this study was within this expected range (8.21 ± 0.27).

Osmolality is also directly related to sperm motility. The sperm of most fish remains quiescent in seminal plasma (~300 mOsm kg^{-1}), and the sperm of freshwater fish is activated upon contact with hypotonic solution (<300 mOsm kg^{-1}; Morisawa and Suzuki, 1980). The present study found consistent results for *P. brevis* (250.29 ± 25.84 mOsm Kg^{-1}). It is therefore very important to adjust the osmolality of the diluent used in cryopreservation to prevent sperm activation prior to freezing.

DMSO has been used successfully for the seminal cryopreservation of several species of Characiformes fish (Godinho and Viveiros, 2011). However, in this study, cryopreservation with DMSO yielded a lower percentage of motile sperm than methylglycol, regardless of the extender used. A similar result was observed by Viveiros *et al*. (2011) for the cryopreservation of *Brycon insignis* semen. One possible cause for this finding is the high toxicity of DMSO at high concentrations. Another possibility is that the stabilization time used was too long to protect the sperm against cryodamage.

An influx of water into the sperm may also occur, as shown in a prior study in which *Cyprinus carpio* sperm swelled as the concentration of DMSO was increased from 1 to 20% (Perchec-Poupard *et al*., 1997). The DMSO concentration used in the present study was 10%.

Previous studies have shown that glucose is the extender that is most often used for the cryopreservation of sperm from characiform fish (Salmito-Vanderley *et al*., 2012) because it results in a high percentage of motile spermatozoa, as observed with *P. lineatus* (Viveiros *et al*., 2010) and *P. magdalenae* (Martínez *et al*., 2012). In the present study, compared with glucose, BTS™ resulted in lower total motility in combination with MG and higher total motility in combination with DMSO. Some component of BTS™ (sodium citrate, EDTA, $NaHCO_3$, KCl, or gentamicin) might have negatively affected the sperm during freezing and thawing (Nascimento *et al*., 2010).

In addition to total motility, some studies have observed a relationship between sperm speed, mainly VCL, and fertilization (Viveiros *et al*., 2010). In the present study, the highest VCL and the greatest number of morphologically normal cells were observed in the semen samples cryopreserved using T2. This outcome might be due to the relative nontoxicity of MG (Viveiros *et al*., 2011) compared with other cryoprotectants.

Another important parameter to consider is spermatozoon morphology. In this study, we observed that the combined MG and glucose treatment yielded a greater number of morphologically normal cells, regardless of the freezing method used. The analysis of the damaged spermatozoa found revealed that the most common morphology was a bent tail, and this result was expected based on the studies conducted by Streit Jr *et al*. (2009) on the cryopreservation of *Piaractus mesopotamicus* sperm. Semen with spermatozoon abnormalities exhibit decreased rates of fertilization because the sperm exhibit circular and oscillatory movements (Kavamoto *et al*., 1999). The normal rate of sperm morphological abnormalities recognized by the Brazilian College of Animal Reproduction for mammalian organisms is 30% for bovines and horses and 20% for sheep and pigs. However, the rates in fish have not yet been established (Miliorini, 2006). The results of the present study suggest that acceptable results may be achieved when following established protocols regardless of the diluting solution and freezing method employed.

Dry shippers are used in most studies on the cryopreservation of characiform fish sperm (Godinho *et al*., 2011), and the programmed freezing machine remains underutilized (Carneiro *et al*., 2012; Martínez and Pardo-Carrasco, 2013). The technique used to decrease seminal temperature during cryopreservation can also influence the results. However, a programmed freezing machine might be a more precise alternative than a dry shipper because it does not require extensive control by the operator and it allows a homogeneous and gradual decrease in temperature. In addition to these advantages, programmable freezers are suitable for freezing large amounts of semen straws (Soares and Guerra, 2009), thus facilitating their use in large-scale aquaculture.

P. brevis sperm can be successfully cryopreserved in glucose combined with the cryoprotectant methylglycol using either a dry shipper or a programmed freezing machine. In the present study, these methods resulted in high numbers of motile and morphologically normal spermatozoa.

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References

Araújo SA, Gurgel HCB. 2002. Aspectos da biologia de *Prochilodus cearenses* (Steindachner, 1911) (Characiformes, Prochilodontidae) no açude Itans/Caicó, Rio Grande do Norte. *Rev Bras Zoociênc*, 4:85-96.

Billard R, Cosson J, Crim, LW, Suquet, M. 1995. Sperm physiology and quality. *In*: Bromage NR, Roberts RJ, (Ed.). *Brood Stock Management and Egg and Larval Quality*. Oxford, UK: Blackwell Science. pp. 25-52.

Carneiro, PCF, Segui MS, Ióris-Filho CR, Mikos JD. 2006. Viabilidade do sêmen do Jundiá, *Rhamdia quelen*, armazenado sob refrigeração. *Rev Acad*, 4:11-16.

Carneiro PCF, Azevedo HC, Santos JP, Maria AN. 2012. Cryopreservation of Tambaqui (*Colossoma macropomum*) semen: extenders, cryoprotectants, dilution ratios and freezing methods. *Cryoletters*, 33:385-393.

Carolsfeld J, Harvey B, Godinho HP, Zaniboni-Filho E. 2003. Cryopreservation of sperm in Brazilian migratory fish conservation. *J Fish Biol*, 63:472-481.

Chellappa S, Bueno RMX, Chellappa T, Chellappa NT, Val VMFA. 2009. Reproductive seasonality of the fish fauna and limnoecology of semi-arid Brazilian reservoirs. *Limnologica*, 39:325-329.

Cosson J. 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. *Aquaculture*, 12:69-85.

Felizardo VO, Mello RA, Murgas LDS, Andrade ES, Drumond MM, Rosa PV. 2010. Effect of cryopreservant combinations on the motility and morphology of Curimba (*Prochilodus lineatus*) sperm. *Anim Reprod Sci*, 122:259-263.

Galo JM, Streit Jr. DP, Sirol RN, Ribeiro RP, Digmayer M, Andrade VXL, Ebert AR. 2011. Spermatic abnormalities of Piracanjuba *Brycon orbignyanus* (Valenciennes, 1849) after cryopreservation. *Braz J Biol*, 71:693-699.

Godinho HP, Viveiros ATM. 2011. Current status of sperm cryopreservation of Brazilian Characiform fishes. *In*: Tiersch TR, Green CC (Ed.). *Cryopreservation in Aquatic Species*. Baton Rouge, LA: World Aquaculture Society. pp. 875-884.

Kavamoto ET, Barnabe VH, Campos BES, Andrade EFT. 1999. Anormalidades morfológicas nos espermatozóides do Curimbatá, *Prochilodus lineatus* (Steindachner, 1881) (Osteichthyes, Characiformes, Prochilodontidae). *Bol Inst Pesca*, 25:61-66.

Maria AN, Azevedo HC, Santos JP, Silva CA, Carneiro PCF. 2010. Semen characterization and sperm structure of the Amazon Tambaqui *Colossoma macropomum*. *J Appl Ichthyol*, 26:779-783.

Martínez JG, Tarazona-Morales AM, Pardo-Carrasco SC. 2012. Sperm cryopreservation of freshwater fish Bocachico (*Prochilodus magdalenae*) in DMSO and glucose and its effects on fertilization and hatching efficiency. *Anim Reprod*, 9:19-26.

Martínez JG, Pardo-Carrasco S. 2013. Effect of freezing and thawing rates on sperm motility in Bocachico *Prochilodus magdalenae* (Pisces,Characiformes). *Rev MVZ Córdoba,* 18:3295-3303.

Miliorini A. 2006. Ativadores e concentrações de metanol e dimetilsulfóxido na qualidade do sêmen criopreservado de Curimba (*Prochilodus lineatus*). Lavras, MG: Federal University of Lavras, Minas Gerais, Brazil. 113 pp. MSc Thesis,

Morisawa M, Suzuki K. 1980. Osmolality and potassium ion: their roles in initiation of sperm motility in teleosts. *Science*, 210:1145-1147.

Morisawa S, Morisawa M. 1986. Acquisition of potential for motility in Rainbow trout and chum salmon. *J Exp Biol*, 126:89-96.

Morisawa S, Morisawa M. 1988. Induction of potential for sperm motility by bicarbonate and pH in Rainbow trout and chum salmon. *J Exp Biol*, 136:13-22.

Nascimento AF, Maria AN, Pessoa NO, Carvalho MAM, Viveiros ATM. 2010. Out-of-season sperm cryopreservation in different media of the Amazonian freshwater fish Pirapitinga (*Piaractus brachypomus*). *Anim Reprod Sci*, 118:324-329.

Nascimento MM, Nascimento WS, Chellappa NT, Chellappa S. 2012. Biologia reprodutiva do Curimatã comum, *Prochilodus brevi*s (Characiformes: Prochilodontidae) no açude Marechal Dutra, Rio Grande do Norte, Brasil. *Bio Amaz*, 2:31-43.

Orfão LH, Nascimento AF, Corrêa FM, Cosson J, Viveiros ATM. 2011. Extender composition, osmolality and cryoprotectant effects on the motility of sperm in the Brazilian endangered species *Brycon opalinus* (Characiformes). *Aquaculture*, 31:241-247.

Perchec-Poupard G, Gatti JL, Cosson J, Jeulin C, Fierville F, Billard R. 1997. Effects of extracellular environment on the osmotic signal transduction involved in activation of motility of carp spermatozoa. *J Reprod Fertil*, 110:315-327.

Salmito-Vanderley CSB, Vieira MJAF, Leite LV, Oliveira FCE, Linhares FRA, Salgueiro CCM, Nunes JF. 2012. Meios de congelação para conservação de sêmen de peixes da família Characideae. *Cienc Anim*, 22:255-268.

Salmito-Vanderley CSB, Pinheiro JPS, Almeida PS, Lopes JT, Leite LV. 2014. Metodologias para criopreservação e mecanismos de avaliação do sêmen de peixes Characiformes. *Acta Vet Bras*, 8:343-350.

Soares AT, Guerra MMP. 2009. Efeitos da criopreservação sobre a viabilidade espermática. *Rev Tecnol Ciênc Agropec*, 3:53-63.

Streit Jr DP, Oliveira AC, Ribeiro RP, Sirol RN,

Moraes GV, Galo JM, Digmayer M. 2009. Motilidade, vigor e patologias seminal in natura e pós criopreservação de *Piaractus mesopotamicus. Bol Inst Pesca*, 35:159-167.

Tabares J, Tarazona A, Oliveira M. 2005. Fisiología de la activación del espermatozoide en peces de agua dulce. *Rev Col Cienc Pec*, 18:149-160.

Viveiros ATM, Nascimento AF, Orfão LH, Isaú ZA. 2010. Motility and fertility of the subtropical freshwater fish Streaked prochilod (*Prochilodus lineatus*) sperm cryopreserved in powdered coconut water. *Theriogenology*, 74:551-556.

Viveiros ATM, Amaral TB, Orfão LH, Isaú ZA, Caneppel D, Leal MC. 2011. Sperm cryopreservation of Tiete tetra *Brycon insignis* (Characiformes): effects of cryoprotectants, extenders, thawing temperatures and activating agents on motility features. *Aquacult Res*, 42:858-865.