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Dimethylsulfoxide, methanol and methylglycol in the seminal cryopreservation of Suruvi, *Steindachneridion scriptum*

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ABSTRACT

The aim of this study was to analyze the effect of 5%, 7.5%, 10%, 12.5% and 15% dimethylsulfoxide (DMSO), methanol and methylglycol alcohols on the cryopreservation of sperm from *Steindachneridion scriptum*. Male specimens ($n = 15$) were obtained from Pisciculture and sperm samples were collected by abdominal massage. Post collection the fresh sperm sample was diluted in the Beltsville Thawing Solution and sperm motility was evaluated. Results indicated that the most precise parameters for total and progressive motility were obtained with the use of methylglycol (all concentrations) and 7.5% and 10% methanol ($P < 0.05$). The motility of the sperm was sustained for the longest time period when 5%, 7.5% and 15% DMSO was used; similar results were also seen for 5% methanol and methylglycol at 7.5%, 10%, 12.5%, and 15% concentration ($P < 0.05$). With respect to reactive oxygen species it was observed that the production of ROS decreased only in presence of 5% methylglycol but not when DMSO (5%) was used ($P < 0.05$). Although the use of methanol (12.5%) allowed for a lesser membrane fluidity as compared to DMSO 12.5% ($P < 0.05$), membrane functional integrity was greater with 10% and 12.5% DMSO ($P < 0.05$) as compared to 10% methanol or 5% methylglycol ($P > 0.05$). Additionally, when major mitochondrial functionalities were assessed it was observed that the values obtained with use of 12.5% and 15% DMSO were comparable to all except 5% methylglycol ($P < 0.05$). In conclusion, the results of the present study indicate that 7.5% methylglycol was the most effective treatment for the cryopreservation of the *S. scriptum* sperm.

1. Introduction

Steindachneridion scriptum (Miranda Ribeiro, 1918), popularly known as Suruvi, is a fish that is found in the Uruguay River and the Upper Rio Paraná (Garavello, 2005). This species is characterized by reproductive migration along with external fertilization and

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total spawning (Britto et al., 2008). Due to dam constructions, and fragmentation of migration rivers there has been a decrease in fish density as well as habitat quality (ICMBio, 2014), this fish is in imminent danger of extinction. For this reason, it is necessary to undertake urgent measures to allow for its preservation.

According to studies by Schork et al. (2013), *S. scriptum* represent the fourth and sixth most captured species in Brazil. This species is also relevant for fish farming because of its docile behavior, and thus lesser stress when there are handling procedures, resistance to low temperatures and great acceptance in the market. Among the conservation possibilities, *ex situ* is regarded as a highly valuable preservation technique with tremendous potential. This technology enables the creation of germplasm banks that function on the principle of cryopreservation biotechnology. The use of cryopreservation allows for the protection of genetic resources and conservation of the biology (Figueroa et al., 2014) making it possible to avoid the loss of any species at the risk of extinction.

One of the drawbacks of cryopreservation is that there may be damage due to heat shock (Bucak et al., 2008) and/or rapid metabolism reestablishment upon sperm cell rehydration (Holt, 2000). Another potential risk factor is freezing may result in cryoinjury wherein the formation of internal and external ice crystals cause mechanical damage to the cellular organelles (Cabrita et al., 2005) as well as cause a loss of sperm motility (Chatterjee et al., 2001). To minimize such damage, diluted solutions of cryoprotectants are added for protecting the spermatozoa (Squires et al., 1999). These cryoprotectants can be divided into two classes: (a) permeable: those that have lesser molecular weights and greater solubility in water allowing for the rapid penetration into the cell and, (b) non-permeable: those that have a greater molecular weight that do not penetrate, and function by protecting the cell externally.

Among the various permeable cryoprotectants that are known, alcohols such as dimethyl sulfoxide (DMSO), methanol and methylglycol have been previously studied in Brazilian fresh water fish for reducing cryoinjury caused by the cryopreservation procedure. For species that are native to Brazil, 5% to 15% DMSO is widely regarded as the most effective cryoprotectant (Viveiros et al., 2015). There, however, are studies from which results indicate methylglycol is more ideally suited for the preservation of spermatozoa of a test species (*Brycon orbignyanus, Leporinus obtusidens* and *Prochilodus lineatus*), because of the greater post-thawing sperm motilities when compared with use of DMSO. A 10% methanol solution in combination with 15% milk powder proved to be highly effective in the cryopreservation of the sperm cells of the native species, *P. coruscans* (Carolsfeld et al., 2003).

Sperm of different species may have varying tolerances for toxicity hence the freezing protocol employed for cryoprotection must be tailored to the species concerned. To the best of our knowledge, no form or protocol has been previously reported in the scientific literature for the freezing of *S. scriptum* sperm.

The objective of the present study was to analyze different concentrations of alcohols (DMSO, methanol and methylglycol: 5%, 7.5%, 10%, 12.5% and 15%) for the cryopreservation of *S. scriptum* sperm.

2. Materials and methods

Sperm samples obtained from *S. scriptum* were subjected to an initial analysis subsequent to which sperm were frozen as part of the cryopreservation process in Paulo Lopes – SC – Brazil (27° 57′37.8″S and 48° 45′29.0″ W). Thawing and other analyses relevant to this study were conducted at the Federal University of Pelotas-UFPEL in Pelotas-RS-Brazil and at the Federal University of Rio Grande-FURG in Rio Grande-RS-Brazil. The study was conducted with authorization from the Ethics Commission for Animal Use (CEUA) of the Federal University of Rio Grande (Ata 005/2016).

2.1. Sperm collection

Fifteen sexually mature wild *S. scriptum* males (2.5 ± 2.0 kg) that were in a period (December) when reproduction was occurring were used for this study. The reproductive period of *S. scriptum* is from late spring (November) until March, with a reproductive peak in December. All subjects were kept in tanks with water that were maintained at a constant 26 °C. For the purpose of the study, all subjects were then hormonally induced using carp pituitary extract (0.5 mg/kg). Twelve hours after inducing, each animal was removed from the tank and dried with a paper towel prior to performing the abdominal massage. Aliquots of the collected semen were stored in 15 mL conical tubes and maintained at a temperature between 5 and 8 °C. Extreme care was taken to ensure that samples were not contaminated by water, feces, blood or urine.

Sperm activation was performed by adding 1 μL of sample to 4 μL of distilled water. Sperm evaluation was performed in fresh semen samples by examining the activated sperm using a 400x contrast optical microscope (Olympus 41 BX) (Carneiro et al., 2012).

2.2. Cryopreservation

The base extender used for the samples in this study was the Beltsville Thawing Solution (BTS) which is composed of 37 g glucose, 6 g sodium citrate, 1.25 g sodium bicarbonate, 1.25 g EDTA, and 0.7 g streptomycin to 1 L distilled water (Pursel and Johnson, 1975). The treatments were: DMSO, methanol (MET), and methylglycol (MG) at the concentrations of 5%, 7.5%, 10%, 12.5% and 15%. All chemicals used in this study were obtained from the Sigma Chemical Company® (St. Louis, MO, USA).

After dilution, the samples were homogenized, packed in 250 μL containers and closed with polyvinyl alcohol. The final dilution resulted in a sperm concentration of 150×10^6 sperm/mL. The samples were then placed in aluminum racks so as to ensure maximal contact between components, and subjected to 20 °C for 20 min. Subsequently, samples were placed in a dry shipper of nitrogen vapor (Taylor-Wharton, model CP 300 dry shipper) for 12 h before being transferred to a liquid nitrogen canister (MVE ™, VOLTA 34, GA, USA) maintained at −196 °C, where samples were stored for at least 30 days (Varela et al., 2012).The samples were thawed by

placing them in a water bath set at 45 °C for 8 s and transferred the thawed contents to 1.5 mL microtubes (Streit et al., 2006).

2.3. System of computerized sperm analysis (CASA)

Sperm samples were activated by mixing 1 μL of sample with 4 μL of distilled water. Sperm kinetics analyses were conducted by examining the activated sperm using a microscope with samples being placed on a slide and a coverslip being applied (Carneiro et al., 2012). A total of 10 fields were assessed within 5 to 10 s of the activation process and a minimum of 1000 cells were analyzed. The variables evaluated were: total motility (%), progressive motility (%), VCL (curvilinear velocity, μm/s), VSL (straight linear velocity, μm/s), VAP (average path velocity, μm/s), LIN (Linearity, [VSL/VCL]x100), mean distance traveled DAP (μm), curvilinear distance DCL (μm), rectilinear distance DSL (μm), velocity (VSL/VAP%), lateral displacement of the head ALH (μm) and rectilinear velocity VSL Crossed BCF (Hz) (Dziewulska et al., 2011). Motility time (TMot) was regarded as the time starting from sperm activation until the end of the progressive movement (Varela et al., 2015).

2.4. Flow cytometry

The Attune Acoustic Focusing® equipment (Life Technologies) was used for flow cytometric analysis using the blue (Argon 488 nm) and violet (UV 405 nm) lasers. The latter was used for the analysis of the following cellular structures: DNA fragmentation, membrane fluidity, membrane and mitochondrial functionality, Lipid Peroxidation (LPO), cellular disruption, and concentration of reactive oxygen species (ROS). The results were obtained using the software version 2.1 (Life Technologies). The Hoechst 33,342 dye at 16.2 mM was used for all flow cytometric analysis except for DNA fragmentation. To eliminate non-spermatic events, FSCxSSC and Hoeschst 33,342 negative scatter plots were generated (Alves et al., 2016).The cells were stained with fluorophores that had been added to calcium-free PBS (0.2 g KCl, 0.2 g KH2PO4, 1.15 g Na2HPO4 and 8 g NaCl in 1 L deionized water, pH 7.2) and a total of 10,000 sperm were counted per analysis (excluding debris).

2.5. Membrane functionality and cellular disruption

Membrane functionality was evaluated using the fluorophores propidium iodide (PI) and Sybr14 (Minitube,Tiefenbach, Germany). An aliquot of thawed semen was incubated for 10 min in a solution containing 0.25 μM of Sybr14 and 7.5 μM IP (as per manufacturer's instructions–Minitube). Spermatozoa were classified as non-damaged with functional membrane (Sybr + /IP-) or damaged with/without a non-functional membrane (Sybr + /IP + , Sybr-/IP + , Sybr-/IP-) (Figueroa et al., 2014). To verify the percentage of cellular rupture, the cells that were IP − were classified as non–ruptured whereas those that were IP + were regarded as ruptured.

2.6. Membrane fluidity

Membrane fluidity was verified by incubation 10 μL of sample in 2.7 μM of hydrophobic merocyanin 540 (M540) dye and 0.1 μM of YO PRO–1 (Invitrogen-Eugene, OR, USA) for 10 min. High-fluid (high concentration of M540) and low fluidity (low concentration of M540) cells were evaluated only for the presence of intact spermatozoa (YO-PRO negative) (Fernández-Gago et al., 2013). The membrane flow rate was calculated using the following formula: [(number of spermatozoa with high fluidity)/(number of spermatozoa with high fluidity) + (spermatozoa with low fluidity)] × 100.

2.7. Mitochondrial functionality

To assess mitochondrial functionality, 10 μL of thawed sample was incubated with 3.1 μM Rhodamine 123 (green fluorescence) and 7.5 μM IP for 10 min. Sperm cells were classified as having either high functionality (high fluorescence by Rhodamine accumulation) or low functionality (low fluorescence, low Rhodamine accumulation) after they had been analyzed for the presence of intact spermatozoa (IP negative) (Liu et al., 2015). The mitochondrial functionality rate was calculated by the following formula: [(number of spermatozoa with high mitochondrial membrane potential)/(high sperm count potential of mitochondrial membrane + spermatozoa with low mitochondrial membrane potential)] × 100 (Alves et al., 2016).

2.8. DNA fragmentation index

DNA integrity was assessed by the chromatin structure assay (SCSA). To verify this variable, 10 μL of thawed spermatozoa were added to 5 μL of TNE (0.01 M Tris − HCl, 0.15 M NaCl, 0.001 M EDTA, pH 7.2) and 10 μLof 1X Triton (Triton X–100, 1%) (V/V) at 30 s intervals. Acridine orange dye was then added to the above. This was followed by a short incubation period of between 30 s to 2 min after which the results were analyzed. Spermatozoa with fragmented DNA presented with red fluorescence, while those with intact DNA exhibited green fluorescence (Evenson and Jost, 2001). The DNA fragmentation rate index (DFI%) was calculated as follows: (red fluorescence)/[total fluorescence intensity (red + green)] (Alves et al., 2016).

2.9. Concentration of reactive oxygen species (ROS)

The ROS concentration was determined by adding 1.0 μM of 2′7′dichlorofluorescein diacetate (H2DCFDA) (emits green fluorescence when oxidized) and 7.5 μM IP to 10 μL of thawed sample followed by an incubation for 10 min. Production of ROS was measured in terms of medium green fluorescence intensity in living cells with negative PI, thus excluding cells with ruptured membranes (Domínguez-Rebolledo et al., 2011).

2.10. Lipid peroxidation (LPO)

Lipid peroxidation of spermatozoa was evaluated immediately after thawing. Bodipy C11 (Hagedorn et al., 2012) was added to 10 μL of sample to a final concentration of 1 μM, and incubated for 2 h at 20 °C. Only live spermatozoa were analyzed. The rate of lipid peroxidation was calculated as follows: [(median intensity of green fluorescence (peroxidized lipid))/(median green fluorescence intensity + median red fluorescence (non-peroxidized lipid))] * 100 (Alves et al., 2016).

2.11. Statistical analysis

All variables were analyzed for normality by the Shapiro-Wilk test followed by analysis of variance (ANOVA) by Tukey's test. The different cryoprotectants and their concentrations were regarded as independent variables while all other variables such as total motility, progressive motility, motile time, DAP, DCL, DSL, VAP, VCL, VSL, STR, LIN, ALH, BCF, membrane functionality, mitochondrial functionality, membrane fluidity, DNA fragmentation index, ROS, LPO and cellular disruption were considered as dependent variables. All analytical procedures were performed using Statistix 2010software. The $P < 0.05$ value was considered to indicate significant differences.

3. Results

The mean (± S.E.M.) of sperm motility for fresh semen was 97.6 ± 1.8% and the motility period 78.4 ± 4.2 s. The most desirable values for total and progressive motility in thawed semen were obtained with use of all concentrations of methylglycol (5%, 7.5%, 10%, 12.5%, and 15%) and for methanol at concentrations of 7.5% and 10% ($P < 0.05$; Table 1). For the treatments with DMSO (5%, 7.5% and 15%), 5% methanol and methylglycol (7.5%, 10%, 12.5%, and 15%, there was the greatest sperm motility time when compared to the other treatments (Table 1).

Analysis of various kinetic variables (total motility, progressive motility, average distance traveled, curvilinear distance, rectilinear distance, mean velocity, curvilinear speed, rectilinearity, oscillation) provided results that indicated the most desirable motility for thawed spermatozoa occurred when 5%, 7.5% and 10% methylglycol was used for treatment purposes ($P < 0.05$; Tables 1 and 2).

The ROS production was less in samples that had been treated with 5% methylglycol as compared with samples that had been treated with 5% DMSO ($P < 0.05$; Table 3). Lipid peroxidation (LPO) and DNA fragmentation index variables, however, were not

Table 1
Analyses of total motility, progressive motility, motility time, mean distance traveled, curvilinear distance, rectilinear distance, and mean velocity of the thawed spermatozoa of *Steindachneridion scriptum* (Miranda-Ribeiro, 1918) with the dimethylsulfoxide, methanol and methylglycol alcohols at different concentrations (mean ± standard error).

	Sperm kinetics						
Treatments (%)	MotTot(%)	MotProg (%)	TMot (s)	DAP (μm)	DCL (μm)	DSL (μm)	VAP (μm/s)
DMSO 5	29.7 ± 1.12 ^{bcd}	22.4 ± 1.07 ^{bcde}	67.8 ± 2.65^{a}	13.4 ± 0.33 ^{bcd}	16.5 ± 0.34 ^{cde}	11.0 ± 0.32 ^{bcde}	29.7 ± 0.73 ^{bcd}
DMSO 7.5	28.7 ± 1.33 ^{cd}	21.6 ± 1.21 ^{de}	55.1 ± 2.81^{abcd}	13.2 ± 0.38 ^{cde}	16.4 ± 0.43 ^{cdef}	10.9 ± 0.36 ^{cdef}	29.2 ± 0.84 ^{bcde}
DMSO 10	31.1 ± 1.54 ^{bcd}	23.2 ± 1.47 ^{cde}	49.1 ± 3.73^{bcde}	12.1 ± 0.30 ^{de}	15.0 ± 0.34 ^{defg}	9.8 ± 0.28 ^{def}	26.5 ± 0.70 ^{de}
DMSO 12.5	24.4 ± 1.25 ^{de}	17.5 ± 1.05 ^{ef}	45.4 ± 2.88^{def}	12.2 ± 0.26 ^{cde}	14.9 ± 0.32 ^{defg}	10.1 ± 0.24 ^{cdef}	26.8 ± 0.63 ^{cde}
DMSO 15	22.2 ± 1.33 ^{e}	15.6 ± 1.20 ^{f}	52.4 ± 3.00 ^{abcd}	12.0 ± 0.25 ^{de}	14.6 ± 0.28 ^{efg}	9.8 ± 0.23 ^{def}	26.8 ± 0.55 ^{cde}
MET 5	32.3 ± 1.65 ^{bcd}	23.7 ± 1.45 ^{cde}	59.5 ± 2.58^{abcd}	13.4 ± 0.33 ^{bcd}	16.8 ± 0.39 ^{bcd}	11.1 ± 0.31 ^{bcd}	29.6 ± 0.75 ^{bcd}
MET 7.5	39.0 ± 2.08 ^{abc}	31.1 ± 1.97 ^{abcd}	49.1 ± 3.44^{cde}	14.3 ± 0.48 ^{bc}	18.0 ± 0.57 ^{bc}	12.0 ± 0.46 ^{bc}	31.5 ± 1.09 ^{bc}
MET 10	42.1 ± 2.15 ^{ab}	31.7 ± 2.03 ^{abcd}	48.1 ± 3.48^{cde}	13.8 ± 0.52 ^{cde}	17.3 ± 0.59 ^{cde}	11.5 ± 0.48 ^{cde}	30.1 ± 1.17 ^{cde}
MET 12.5	26.1 ± 1.34 ^{de}	18.6 ± 1.20 ^{ef}	30.9 ± 2.83 ^{f}	11.8 ± 0.32 ^{e}	14.3 ± 0.37 ^{fg}	9.8 ± 0.30 ^{ef}	25.9 ± 0.71 ^{e}
MET 15	26.8 ± 1.47 ^{de}	19.4 ± 1.28 ^{ef}	34.8 ± 3.54 ^{ef}	11.4 ± 0.24 ^{e}	14.0 ± 0.29 ^{g}	9.4 ± 0.23 ^{f}	25.3 ± 0.55 ^{e}
MG 5	44.5 ± 2.16 ^{a}	35.7 ± 2.05 ^{a}	47.6 ± 4.12 ^{bcde}	17.9 ± 0.67 ^{a}	22.6 ± 0.69 ^{a}	15.4 ± 0.63 ^{a}	39.9 ± 1.61 ^{a}
MG 7.5	44.1 ± 2.30 ^{a}	36.5 ± 2.24 ^{a}	64.5 ± 3.93 ^{ab}	18.6 ± 0.71 ^{a}	22.9 ± 0.72 ^{a}	16.2 ± 0.71 ^{a}	40.9 ± 1.54 ^{a}
MG 10	47.3 ± 2.15 ^{a}	37.7 ± 2.04 ^{a}	60.6 ± 2.68 ^{abc}	15.4 ± 0.49 ^{ab}	19.4 ± 0.54 ^{ab}	13.2 ± 0.47 ^{ab}	33.9 ± 1.17 ^{ab}
MG 12.5	43.2 ± 2.23 ^{a}	32.7 ± 2.19 ^{abc}	54.5 ± 3.24 ^{abcde}	14.2 ± 0.56 ^{bcd}	18.2 ± 0.59 ^{bc}	11.8 ± 0.55 ^{bcde}	31.6 ± 1.29 ^{bcd}
MG 15	42.9 ± 1.70 ^{a}	31.5 ± 1.57 ^{ab}	49.5 ± 3.01 ^{abcde}	12.5 ± 0.32 ^{cde}	16.0 ± 0.37 ^{cdef}	10.4 ± 0.31 ^{cdef}	27.3 ± 0.70 ^{cde}

Total motility (MotTot), progressive motility (MotProg),Time of motility (TMot),Mean distance traveled (DAP), curvilinear distance (DCL), rectilinear distance (DSL), average path velocity (VAP), dimethylsulfoxide (DMSO), methanol (MET) and methylglycol (MG). Different letters in the same column indicate differences ($P < 0.05$).

Table 2
Curvilinear velocity (VCL), linear velocity (VSL), straightness (STR), linearity (LIN), lateral head displacement (ALH), frequency of cross flagellar beating (BCF) of the thawed spermatozoa of *Steindachneridion scriptum* (Miranda-Ribeiro, 1918) with the dimethylsulfoxide (DMSO), methanol (MET) and methylglycol (MG) alcohols at different concentrations (Mean ± standard error).

	Analyses of sperm kinetics					
Treatments (%)	VCL (μm/s)	VSL (μm/s)	STR (%)	LIN (%)	ALH (μm)	BCF (Hertz)
DMSO 5	36.5 ± 0.78 ^{bcd}	24.2 ± 0.70 ^{bcde}	0.81 ± 0.005 ^{d}	0.65 ± 0.008 ^{ab}	1.53 ± 0.05 ^{a}	23.1 ± 0.32 ^{def}
DMSO 7.5	36.3 ± 1.01 ^{cd}	24.0 ± 0.79 ^{cdef}	0.81 ± 0.006 ^{bcd}	0.66 ± 0.009 ^{ab}	1.50 ± 0.07 ^{abc}	22.7 ± 0.44 ^{defg}
DMSO 10	33.1 ± 0.81 ^{de}	21.5 ± 0.62 ^{def}	0.80 ± 0.005 ^{d}	0.65 ± 0.009 ^{ab}	1.36 ± 0.06 ^{abc}	21.5 ± 0.38 ^{efg}
DMSO 12.5	33.0 ± 0.82 ^{de}	22.1 ± 0.54 ^{cdef}	0.82 ± 0.005 ^{bcd}	0.67 ± 0.001 ^{ab}	1.42 ± 0.07 ^{abc}	22.7 ± 0.53 ^{defg}
DMSO 15	32.8 ± 0.67 ^{de}	21.7 ± 0.48 ^{cdef}	0.81 ± 0.005 ^{d}	0.66 ± 0.009^{ab}	1.57 ± 0.06 ^{ab}	21.8 ± 0.37 ^{efg}
MET 5	37.1 ± 0.89 ^{bcd}	24.5 ± 0.67 ^{bcd}	0.82 ± 0.005 ^{bcd}	0.66 ± 0.009 ^{ab}	1.43 ± 0.05 ^{abc}	23.6 ± 0.39 ^{de}
MET 7.5	39.5 ± 1.27 ^{bc}	26.3 ± 1.01 ^{bc}	0.82 ± 0.005 ^{abcd}	0.66 ± 0.009 ^{ab}	1.41 ± 0.06 ^{abc}	24.7 ± 0.58 ^{cd}
MET 10	37.9 ± 1.35 ^{cd}	25.1 ± 1.07 ^{cdef}	0.82 ± 0.005^{bcd}	0.66 ± 0.009 ^{ab}	1.26 ± 0.04 ^{bc}	23.8 ± 0.53 ^{de}
MET 12.5	31.3 ± 0.84 ^{e}	21.5 ± 0.65 ^{ef}	0.82 ± 0.005 ^{bcd}	0.68 ± 0.009 ^{a}	1.26 ± 0.05 ^{c}	21.0 ± 0.47 ^{fg}
MET 15	30.9 ± 0.69 ^{e}	20.6 ± 0.50 ^{f}	0.82 ± 0.005 ^{d}	0.67 ± 0.009 ^{ab}	1.34 ± 0.06 ^{abc}	21.0 ± 0.44 ^{g}
MG 5	50.3 ± 1.66 ^{a}	34.2 ± 1.50 ^{a}	0.84 ± 0.006 ^{abc}	0.66 ± 0.011 ^{ab}	1.55 ± 0.07 ^{abc}	28.7 ± 0.50 ^{ab}
MG 7.5	50.5 ± 1.56 ^{a}	35.6 ± 1.53 ^{a}	0.85 ± 0.006 ^{a}	0.68 ± 0.011 ^{ab}	1.55 ± 0.06 ^{ab}	30.8 ± 0.57 ^{a}
MG 10	42.6 ± 1.27 ^{ab}	28.9 ± 1.10 ^{ab}	0.84 ± 0.004 ^{ab}	0.68 ± 0.008 ^{ab}	1.34 ± 0.05 ^{abc}	26.7 ± 0.49 ^{bc}
MG 12.5	40.5 ± 1.36 ^{bc}	26.1 ± 1.23 ^{bcde}	0.81 ± 0.007 ^{bcd}	0.63 ± 0.011 ^{b}	1.48 ± 0.07 ^{abc}	24.3 ± 0.52 ^{cde}
MG 15	35.0 ± 0.82 ^{cde}	22.5 ± 0.66 ^{cdef}	0.82 ± 0.005 ^{cd}	0.64 ± 0.009 ^{ab}	1.28 ± 0.05 ^{abc}	22.9 ± 0.44 ^{defg}

Different letters in the same column indicate differences ($P < 0.05$).

Table 3
Analyses of the sperm organelles (mitochondrial functionality, membrane functionality, cell integrity, membrane fluidity, and DNA fragmentation) and biochemistry (reactive oxygen species and lipid peroxidation) of the thawed spermatozoa of *Steindachneridion scriptum* (Miranda-Ribeiro, 1918) with dimethylsulfoxide (DMSO), methanol (MET) and methylglycol (MG) alcohols at different concentrations (mean ± standard error).

	Analyses						
Treatments (%)	FMit (%)	FMe (%)	IC (%)	FM (%)	DNA (%)	ROS (%)	LPO (%)
DMSO 5	47.6 ± 2.4 ^{ab}	40.5 ± 5.0 ^{ab}	51.8 ± 4.4 ^{a}	46.7 ± 1.9 ^{abc}	0.042 ± 0.003 ^{a}	9723.6 ± 3553.7 ^{a}	54.4 ± 6.4 ^{a}
DMSO 7.5	44.5 ± 2.5 ^{ab}	43.5 ± 5.0 ^{ab}	48.2 ± 4.5 ^{a}	43.1 ± 2.2 ^{abc}	0.044 ± 0.003 ^{a}	1822.5 ± 728.7 ^{ab}	53.7 ± 5.9 ^{a}
DMSO 10	49.1 ± 2.8 ^{ab}	50.4 ± 4.9 ^{a}	52.8 ± 4.8 ^{a}	48.9 ± 2.4 ^{abc}	0.051 ± 0.005 ^{a}	6838.8 ± 2696.4^{ab}	54.5 ± 6.3 ^{a}
DMSO 12.5	51.0 ± 3.1 ^{a}	53.0 ± 5.0 ^{a}	48.6 ± 6.7 ^{ab}	50.8 ± 1.6 ^{a}	0.042 ± 0.002 ^{a}	4197.2 ± 2469.4 ^{ab}	45.3 ± 6.3 ^{a}
DMSO 15	51.9 ± 3.2 ^{a}	48.0 ± 5.0 ^{ab}	42.7 ± 3.3 ^{ab}	50.4 ± 2.3 ^{ab}	0.056 ± 0.011 ^{a}	983.0 ± 275.9 ^{ab}	44.5 ± 6.3 ^{a}
MET 5	42.4 ± 2.7^{ab}	33.0 ± 4.7 ^{ab}	46.7 ± 3.5 ^{ab}	46.3 ± 1.5 ^{abc}	0.046 ± 0.003 ^{a}	4960.8 ± 2350.2 ^{ab}	51.4 ± 5.8 ^{a}
MET 7.5	40.8 ± 2.6 ^{ab}	30.4 ± 5.9 ^{ab}	38.4 ± 3.5 ^{ab}	45.8 ± 2.3 ^{abc}	0.046 ± 0.004 ^{a}	1757.6 ± 717.4 ^{ab}	47.8 ± 5.8 ^{a}
MET 10	42.2 ± 2.7 ^{ab}	25.9 ± 5.0 ^{b}	41.7 ± 2.8 ^{ab}	42.0 ± 2.1 ^{abc}	0.039 ± 0.002 ^{a}	655.3 ± 196.9 ^{ab}	55.0 ± 6.1 ^{a}
MET 12.5	46.3 ± 3.6 ^{ab}	32.8 ± 5.6 ^{ab}	44.6 ± 3.5 ^{ab}	38.6 ± 1.5 ^{c}	0.043 ± 0.003 ^{a}	3764.8 ± 1638.7 ^{ab}	49.2 ± 6.7 ^{a}
MET 15	41.4 ± 3.1 ^{ab}	30.6 ± 4.7 ^{ab}	40.9 ± 3.5 ^{ab}	43.9 ± 2.3 ^{abc}	0.051 ± 0.007 ^{a}	1594.3 ± 595.4 ^{ab}	46.8 ± 5.8 ^{a}
MG 5	35.1 ± 2.6 ^{b}	22.5 ± 3.8 ^{b}	27.5 ± 4.2 ^{b}	40.2 ± 1.5 ^{bc}	0.038 ± 0.001 ^{a}	345.9 ± 87.7 ^{b}	48.5 ± 5.7 ^{a}
MG 7.5	37.6 ± 2.8 ^{ab}	28.2 ± 4.0 ^{ab}	33.8 ± 4.3 ^{ab}	41.9 ± 2.2 ^{abc}	0.036 ± 0.001 ^{a}	4298.9 ± 1919.8 ^{ab}	48.8 ± 6.7 ^{a}
MG 10	39.7 ± 2.7 ^{ab}	36.2 ± 4.4 ^{ab}	32.3 ± 4.2 ^{ab}	43.7 ± 1.9 ^{abc}	0.043 ± 0.003 ^{a}	1064.0 ± 338.1 ^{ab}	45.7 ± 6.3 ^{a}
MG 12.5	41.6 ± 3.0 ^{ab}	33.5 ± 4.4 ^{ab}	38.4 ± 4.3 ^{ab}	40.4 ± 1.9 ^{abc}	0.038 ± 0.001 ^{a}	1639.8 ± 696.7 ^{ab}	33.1 ± 5.5 ^{a}
MG 15	44.8 ± 2.9 ^{ab}	34.8 ± 4.6 ^{ab}	41.7 ± 4.3 ^{ab}	44.7 ± 2.0 ^{abc}	0.047 ± 0.005 ^{a}	1045.0 ± 271.2 ^{ab}	44.6 ± 6.2 ^{a}

Mitochondrial functionality (FMit), membrane functionality (FMe), cell integrity (IC), membrane fluidity (FM), DNA fragmentation, reactive oxygen species (ROS) and lipid peroxidation (LPO). Different letters in the same column indicate differences ($P < 0.05$).

observed to differ between the two ($P > 0.05$; Table 3). During thawing of sperm, DMSO use at 5%, 7.5%, and 10% resulted in greater protection with respect to cell integrity as compared to treatment with 5% methylglycol ($P < 0.05$; Table 3).After thawing the sperm cells, the treatment with 12.5% methanol, resulted in a lesser flow cytoplasmic membrane measurement with greater preservation of membrane permeability as compared with use of 12.5% DMSO ($P < 0.05$; Table 3). When the membrane functionality of these spermatozoa was evaluated, treatment with 10% and 12.5% DMSO ($P < 0.05$) resulted in greater functionality as compared with 10% methanol or 5% methylglycol ($P > 0.05$; Table 3).

4. Discussion

Sperm motility is the most important variable that is assessed for evaluating sperm quality after thawing (Maria et al., 2006). Motility is essential for ensuring reproductive success (Cabrita et al., 2010) as sperm movement propels the spermatozoa towards the oocyte for fertilization purposes. To the best of our knowledge, this is the first study of its kind aimed at evaluating variables related to retention of motility and function after sperm preservation in the Brazillian native species *S. scriptum*. In the present study, it was observed that cryopreservation of sperm with 7.5% methylglycol yielded the most desirable results after thawing in terms of sperm movement.

The duration of motility is another fundamental factor that influences reproductive success. According to Billard (1986), the duration of motility usually ranges from 30 to 60 s for freshwater species with external fertilization. Based on this previous result, it can concluded that the values determined for the Suruvi sperm both as fresh (78 s) as well as for thawed samples (30–67 s) are adequate for reproduction purposes. Results of the present study indicated that the use of DMSO conferred a greater protection to the membranous organelles (membrane functionality, mitochondrial functionality, and cell integrity) and with its use there was a lesser membrane fluidity during freezing. It is well established that the low temperatures that the samples are exposed to during cryopreservation is one of the main causes of destabilization of proteins and lipids present in the plasma membrane (Hazel and Williams, 1990) besides causing changes to its natural fluidity by modifying the interactions and distributions of the previously described components (Van Meer et al., 2008). The use of a cryoprotectant makes it possible to protect these organelles during the freezing process.

The use of DMSO, which has a very low molecular weight (Lovelock and Bishop, 1959) reduces the formation of ice crystals (Thirumala et al., 2006) and provides a more stable cellular environment during freezing. According to Sojka et al. (1990), DMSO has the capacity to interact or combine with lipids, proteins and other cellular components without irreversibly altering the molecular organization thus allowing for its reestablishment post-thawing.

Production of reactive oxygen species (ROS) during the cryopreservation process induces oxidative stress as well as lipid peroxidation (LPO) both of which can reduce sperm quality (Thomson et al., 2009; Aitken and Curry, 2011; Aitken et al., 2012). Hence, it is essential to reduce the production of ROS so as to minimize cell damage (Shaliutina et al., 2013). When there was use of 5% methylglycol as a cryoprotectant in the present study, there was a lesser amount of reactive oxygen species production when compared with use of 5% DMSO. The oxidative stress resulting from the use of the 5% DMSO treatment during the freezing process, however, did not induce a greater lipid peroxidation in the cells when compared with imposing the other treatments.

Methylglycol, a derivative of methanol (CH3OH) and ethylene oxide (CH2OCH2) (Viveiros et al., 2009b), is regarded as a relatively non-toxic alcohol (Takagi et al., 1993). In addition, this cryoprotectant resulted in the desirable effects on the sperm kinetics (total motility and progressive motility) and when this treatment was imposed sperm had greater motility time after thawing. Maria et al. (2006) also reported that with use of methylglycol as a cryoprotectant there were superior results when compared to use of DMSO or methanol when there was evaluation of thawed sperm motility of Piracanjuba (Maria et al., 2006).

From a study conducted by Viveiros et al. (2009a), it was reported that the use of methylglycol as a cryoprotectant to preserve semen of native species resulted in comparable findings to or sometimes even more desirable outcomes than with use of DMSO. These previous findings are consistent with those from the present study where thawing of the Suruvi sperm cells that had been preserved using methylglycol resulted in greater values for enhanced motility qualities as compared with use of DMSO. Results of the present study also indicate that methanol, at any concentration, was not satisfactory as a cryoprotectant possibly due to its inefficiency in the dehydration and hydration processes involved in the freezing of the sperm cells of Suruvi. Similar findings were also reported by Viveiros et al., 2015 where treatment with methanol reduced the post-thawing sperm quality of Piracanjuba (*Brycon orbignyanus*). Interestingly, Viveiros and Godinho (2009) hypothesized that it is possible that the composition of seminal plasma affects the sensitivity of spermatozoa towards the action of cryoprotectants. This further emphasized the cryopreservation protocol is species-specific and that the concentration of cryoprotectant used or even the base diluent used has the potential to compromise its effectiveness for cryopreservation of sperm.

In conclusion, in the present study the use of 7.5%–10% methylglycol with BTS resulted in maintenance of cell viability and preservation of sperm kinetics upon freezing. Based on these results, it is proposed that this combination of cryoprotectants is an effective treatment for sperm preservation of this endangered species and should be used for the formation of its germplasm bank. In addition, it is emphasized that there is a great sensitivity of these spermatozoa to methanol and recommend that its use should be avoided for preservation purposes. It, therefore, is concluded that for the cryopreservation of *S. Scriptum* sperm, the most desirable results occurred when both kinetics as well as the preservation of the spermatic organelles were considered, with the use of BTS and 7.5% methyl glycol.

Conflicts of interest

The authors declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted. The company that provided the fish strains did not have any financial or any other influence in the study.

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