CRYOPRESERVATION OF *Prochilodus lineatus* SEMEN: EFFECT OF CRYOPROTECTANTS COMBINATION

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ABSTRACT

The study was conducted with the objective of comparing the toxicity and the effect of the combination of intra and extracellular cryoprotectants in curimba *Prochilodus lineatus* sperm cells subjected to cryopreservation. Semen from 19 males were analyzed and diluted in four solutions comprise of intra and extracellular cryoprotectants in the following combinations: methanol+lactose, methanol+egg yolk, DMSO+lactose and DMSO+egg yolk. A portion of the diluted semen was frozen while the remaining fraction was kept in repose and evaluated after 10 min. For freezing, the diluted samples were packaged in 0.50 mL straws and placed into liquid nitrogen vapor for 24 h and, after this time, submerged into liquid nitrogen for 10 days. The combination of DMSO+lactose was less toxic to the diluted semen, resulting in higher motility rates and durations when compared to the other treatments. After thawing, the highest motility rate and duration were obtained using lactose as extracellular cryoprotectant, regardless of its combination. There was no significant difference between treatments when analyzing sperm morphology after thawing. Considering the effects of the tested treatments, the use of lactose as an extracellular cryoprotectant added with DMSO or methanol is the most suitable, since these combinations presented the highest motility rates and durations and low morphological change rate after thawing.

Keywords: genetic preservation; cryobiology; sperm analysis; freezing protocol; toxicity; curimba

CRIOPRESERVAÇÃO DO SÊMEN DO *Prochilodus lineatus*: EFEITO DA COMBINAÇÃO DE CRIOPROTETORES

RESUMO

O estudo foi realizado com o objetivo de comparar a toxicidade e o efeito da combinação de crioprotetoresintra e extracelulares em espermatozóides de curimba *Prochilodus lineatus* submetidos à criopreservação. O sêmen de 19 machos foi analisado e diluído em quatro soluções com crioprotetores intra e extracelulares nas seguintes combinações: metanol+lactose, metanol+gema de ovo, DMSO+lactose e DMSO+gema de ovo. Uma porção do sêmen diluído foi congelada enquanto que a fração remanescente foi mantida em repouso e avaliada após 10 min. Para o congelamento, as amostras diluídas foram envasadas em palhetas de 0,50 mL e colocadas em vapor de nitrogênio líquido durante 24 h e, após este tempo, submersas em nitrogênio líquido durante 10 dias. A combinação de DMSO+lactose se mostrou menos tóxica para o sêmen diluído, resultando em taxas mais elevadas de motilidade e duração espermática quando comparadas com os outros tratamentos. Após descongelamento, as maiores taxas de motilidade e duração foram obtidas usando lactose como crioprotector extracelular, independentemente da sua combinação. Não houve diferença significativa entre os tratamentos ao analisar a morfologia espermática após a descongemento. Considerando-se os efeitos dos tratamentos utilizados, o uso de lactose como crioprotector extracelular, adicionada ao DMSO ou metanol, é o mais adequado, uma vez que estas combinações apresentaram maiores taxas de motilidade e duração espermática, e baixa taxa de alteração morfológica após o descongelamento.

Palavras chave: conservação genética; criobiologia; análise espermática; protocolo de congelamento; toxicidade

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INTRODUCTION

Curimba (*Prochilodus lineatus*) is a teleost fish native of South America, widely used in assisted reproduction. In addition to offering great advantages to fish farming due, this species readily responds to hormonal induction (FRANCISCATTO, 2002; NAVARRO *et al*., 2007). Therefore, the interest in preserving curimba has considerably increased making the development of techniques for this purpose becomes essential.

Cryopreservation is a technique used to preserve the genetic material, storing them in liquid nitrogen (-196 ºC). In addition to being indicated to minimize asynchrony in the maturation of gametes and facilitate their transport, this technique limits the stock of males in intensive fish farming, reducing production costs (MARTÍNEZ-PÁRAMO *et al*., 2009). Cryopreservation is also important to preserve diversity of native species, reducing the negative impacts of the hydroelectric dams in the reproductive cycle.

Despite the cryopreservation present several benefits, during cooling, the sperm cells are exposed to external conditions that damage them, interfering directly in their survival. To prevent this damage, the use of cryoprotectants becomes important in the process of cryopreservation. Such substances are intended to protect the sperm cell during freezing and thawing, preventing criolesions caused by the drop in temperature (SQUIRES *et al.,* 2004).

Intracellular cryoprotectants are used to reduce the formation of microcrystals of ice within the cell, while the extracellular act stabilizing and fixing the outer membrane. Dimethylsufoxide (DMSO) is the most used internal cryoprotectant in freezing semen of freshwater fish native to Brazil due to its low molecular weight and rapid absorption, and it can be combined with extracellular cryoprotectants to increase the protection of sperm cells.

The success of cryopreservation technique depends on the efficiency of the protection of the sperm cells during freezing, and choosing the best cryoprotectant and extender solution to avoid chemical and osmotic toxicity for sperm (GAO *et al.,* 1995; SQUIRES *et al.,* 2004).

This study was conducted with the objective of comparing the toxicity and the effect of the combination of intra and extracellular cryoprotectants in *P. lineatus* sperm cells subjected to cryopreservation.

MATERIAL AND METHODS

Breeding

For this experiment, 19 males of curimba were used with body weights between 910-2050 g. The animals were kept in incubators land in Itutinga Environmental Station, Companhia Energética de Minas Gerais (Cemig - EAI).

The selection of males for the experiment was carried out using a light massage in the coelomic cavity, and animals which released a small amount of semen were transported to aquariums of masonry of 2000 L and maintained at a density of six animals per aquarium. The oxygen level was monitored and the water temperature was maintained at around 28 °C.

All care and welfare of animals, detailed in the protocol nº 039/13 approved in June 27, 2013, are in accordance with the Ethical Principles in Animal Experimentation, adopted by the Bioethic Committee in Animal Use (Standing committees/ PRP-Ufla).

Hormonal treatment

To be subjected to hormonal treatment with crude carp pituitary extract (CCPE), each male was weighed and received intramuscular injections of 0.5 and 5.0 mg kg^{-1} of CCPE per body weight, near the base of the dorsal fin. There was an interval of 12 h between applications and semen collection occurred around 7 h after the last dose of CCPE.

Collection of semen

Before collection, the urogenital papilla was clean with a paper towel and semen was collected in sterile test tubes through light massage in coelomic wall in the craniocaudal direction and the samples were kept at room temperature (23 ºC).

The rate and duration of sperm motility were measured in semen *in natura* depositing

an aliquot of 10 µL of semen from each animal in a histological glass slide and homogenized with 40 µL of distilled water. A quantitative assessment of motility was estimated based on the average percentage of mobile spermatozoa, and duration (in seconds) was measured from mixing with distilled water until only 10% of the cells they met in motion.

For the study of cryopreservation, an aliquot of 400 µL of semen in natura was kept at 26 ± 0.8 °C during the handling and analysis of samples.

Cryopreservation and thawing of semen

Semen samples were diluted 1:5 (100 µL of semen : 500 µL of extender solution). The solutions were prepared using 5% BTS (Belltsville Thawing Solution), 10% of intracellular cryoprotectant (methanol or DMSO) and 8% of extracellular cryoprotectant (egg yolk or lactose) diluted in 100 mL of distilled water. Four combinations of intra and extracellular cryoprotectants were tested: DMSO+egg yolk, DMSO+lactose, methanol+egg yolk and methanol+lactose.

The fresh semen samples were in contact with the cryoprotectant solution for 10 min (stabilization period) and after this time, the rate and duration of motility were measured prior to cryopreservation. Samples containing high motility were packaged in 0.50 mL straws which were sealed with dough sterile surgical. Then, the straws were placed in a tank of vapor of liquid nitrogen (Taylor-Wharton , CP 300 "dry shipper", Theodore, AL, USA) for 24 h, which has standard freezing rate of -35.6 °C min^{-1} between 23 and -170 °C. After this period, samples were immersed directly into cylinder of liquid nitrogen at -196 °C (Cryometal, DS-18, Kolovratska, Czech Republic) for 10 days (MURGAS *et al*., 2007).

After 10 days, the individual straws were thawed by immersion in water (water bath) to 60 °C for 8 sec (MARIA *et al*., 2006; PAULINO *et al*., 2012). A 10 µL aliquot of semen was deposited on a histological slide and homogenized with 40 µL of distilled water for activation of the sperm cells. A quantitative assessment of motility was estimated based on the average percentage of mobile spermatozoa and duration (in seconds) was estimated from homogenization with distilled water until only 10% of the sperm cells were moving.

For morphological analysis, an aliquot of 10 µL of thawed semen was diluted in 990 µL of solution of formaldehyde-citrate and a fraction of 10 µL of the sample was deposited on a histological slide along with 10 µL of Rose Bengal dye 1%. After homogenization, a delicate smear was performed and abnormalities in the head, tail and intermediate part were observed throughout the slide, totaling 100 cells per sample.

Statistical analysis

The experimental design was completely randomized. Analysis of normality for all dependent variables was performed using the Shapiro-Wilk test. The data collected were subjected to analysis of variance and F test; when differences detected between the means, the Tukey test was performed. The statistical program used was SAS version 9.0 (2002) and the level of significance was set at $P<0.05$.

RESULTS

Sperm motility in semen diluted in cryoprotectant solutions

Although the motility rates were lower ($P<0.05$) when compared to fresh semen (Table 1), the semen samples of curimba exhibited higher percentages of motile cells after dilution in solutions. These data demonstrate that these combinations of cryoprotectants were not toxic to curimba semen, reaching an average of 82.43% of motility.

Table 1. Contrast to the rate of motility (%) in fresh semen and diluted of the curimba *Prochilodus lineatus*.

	Average rate motility (%)
Fresh semen	98.53 ± 3.4a
Diluted semen	82.43 ± 14.0b

[a,b] Means (± standard deviation) followed by different letters in columns differ by Tukey (P<0,05).

Table 2 shows a greater rate of motility ($P<0.05$) in treatments using methanol+egg yolk and DMSO+lactose, and a longer duration of motility ($P<0.05$) in treatments using lactose as extracellular cryoprotectant, combined with DMSO (77.59 s) or methanol (80.35 s).

Table 2. Average rate (%) and duration (s) of motility in the curimba *Prochilodus lineatus* semen diluted in the cryoprotectant solutions, prior to freezing (n = 19).

Extracellular cryoprotectant (8%)	Intracellular cryoprotectant (10%)			
	DMSO	METHANOL	DMSO	METHANOL
	Rate (%)		Duration (s)	
EGG YOLK	80.59 ± 9.5bB	90.29 ± 15.9aA	59.88 ± 16.2bA	60.82 ± 18.9bA
LACTOSE	88.24 ± 9.9aA	70.59 ± 20.5bB	77.59 ± 47.1aA	80.35 ± 48.1aA

[a,b] Means (± standard deviation) followed by different letters in columns differ by Tukey (P<0,05); [A,B] Means (± standard deviation) followed by different letters in rows, differ by Tukey (P<0,05).

Sperm motility after in thawing

The highest rates of sperm motility (P<0.05) were found in treatments using lactose as extracellular cryoprotectant, combined with DMSO (55.29%) or methanol (57.35%) (Table 3). The solutions containing egg yolk promoted lower rates of motility (P<0.05) independently of the intracellular cryoprotectant used.

In this study, the average motility rate of all cryoprotectants after thawing decreased 48% when compared to fresh semen and 38% when compared to diluted semen (Table 4).

Table 3. Average rate (%) of motility (%) in the curimba *Prochilodus lineatus* sperm after thawing (n = 19).

Extracellular cryoprotectant (8%)	Intracellular cryoprotectant (10%)	
	DMSO	METHANOL
EGG YOLK	41.18 ± 24.1bB	50.10 ± 25.4bA
LACTOSE	55.29 ± 26.4aA	57.35 ± 26.8aA

[a,b]Means (± standard deviation) followed by different letters in columns differ by Tukey test(P<0,05); [A,B] Means (± standard deviation) followed by different letters in rows, differ by Tukey test (P<0,05).

Table 4. Contrast to the average rate (%) and duration (s) of motility in fresh, thawed and diluted semen of curimba *Prochilodus lineatus* (n = 19).

Semen	Rate ofmotility (%)	Duration of motility (s)
Fresh	98.53 ± 3.4A	90.41 ± 48.7A
Diluted	82.43 ± 14.0B	74.66 ± 35.8B
Thawed	50.98 ± 25.7B	67.88 ± 51.5A

[A,B]Means (± standard deviation) followed by different letters in rows, differ by Tukey test (P<0,05).

For the duration of sperm motility, the highest values (P<0.05) were found in treatments using lactose combined with DMSO (73.94 s) or methanol (74.18 s) (Table 5). Our data demonstrate a significant reduction in the rate and duration of sperm motility (P<0.05) after thawing when using egg yolk as extracellular cryoprotectant.

Sperm morphology after thawing

There was no significant difference between treatments for the analysis of sperm morphology in semen thawed (P>0.05) (Table 6), and the observed changes are detailed in Table 7.

Table 5.Average duration of motility (s) in the curimba*Prochilodus lineatus* sperm after thawing (n = 19).

Extracellular cryoprotectant (8%)	Intracellular cryoprotectant (10%)	
	DMSO	METHANOL
EGG YOLK	59.65 ± 49.2bA	63.76 ± 59.1bA
LACTOSE	73.94 ± 53.1aA	74.18 ± 44.6aA

[a,b]Means (± standard deviation) followed by different letters in columns differ by Tukey test (P<0,05); [A,B] Means (± standard deviation) followed by different letters in rows, differ by Tukey test (P<0,05)

Table 6. Average morphological changes (%), primary and secondary, in curimba *Prochilodus lineatus* semen, after thawing (n = 19) (mean ± standard deviation).

Treatments	Morphological changes (%)		
	Primary	Secundary	Total
DMSO+egg yolk	5.84 ± 5.4	20.76 ± 5.4	26.60 ± 8.0
Methanol+egg yolk	4.48 ± 4.5	19.88 ± 9.0	24.36 ± 10.4
DMSO+lactose	4.96 ± 8.2	20.96 ± 5.2	25.92 ± 11.2
Methanol+lactose	3.88 ± 5.0	20.97 ± 10.0	24.85 ± 13.9

Table 7. Detailed average of morphological changes (%) in curimba *Prochilodus lineatus* semen after thawing (n = 19) (mean ± standard deviation).

	Treatment				
	T1	T2	T3	T4	P*
Primary changes					
Isolated head	5.63 ± 4.5	4.06 ± 3.3	4.29 ± 5.6	3.50 ± 2.6	0.5013
Bent tail	0.21 ± 0.4	0.42 ± 0.6	0.67 ± 1.1	0.38 ± 0.6	0.7568
Secundary changes					
Macrocephaly	1.88 ± 2.3	2.19 ± 2.5	2.18 ± 1.7	1.63 ± 2.6	0.4163
Microcephaly	1.44 ± 1.6	1.25 ± 1.2	2.06 ± 2.0	1.75 ± 1.9	0.5885
Degenerate head	1.31 ± 1.3	0.88 ± 1.2	1.19 ± 1.5	0.65 ± 1.0	0.6692
Degenerate midpiece	2.88 ± 2.2	1.69 ± 1.8	2.41 ± 2.0	2.50 ± 1.8	0.4035
Fractured tail	5.56 ± 3.2	3.56 ± 2.6	5.06 ± 5.4	5.56 ± 5.2	0.3144
Coiled tail	6.50 ± 3.6	9.00 ± 6.0	7.47 ± 4.3	8.00 ± 5.3	0.7243
Degenerate tail	1.19 ± 2.6	1.31 ± 2.1	0.59 ± 1.0	0.88 ± 1.5	0.6697
TOTAL	26.60 ± 8.0	24.36 ± 10.4	25.92 ± 11.2	24.85 ± 13.9	0.7607

T1 = DMSO+egg yolk; T2 = methanol+egg yolk; T3 = DMSO+lactose; T4 = methanol+lactose.
** Probability according to the Kruskal-Wallis test at 5%.*

DISCUSSION

The dilution of semen is a necessary step in cryopreservation due to the high sperm concentration in semen. However, this process reduces constituents of the seminal plasma, making the sperm more sensitive to oxidative damage (MARTÍNEZ-PÁRAMO *et al.*, 2012).

In this study, the choice of cryoprotectants was based on their physicochemical properties (molecular weight and toxicity), protection capacity, and their popularity in scientific research.

In tests using 7.5% methanol and DMSO, MILIORINI (2006) obtained, respectively, 87 and 90% of motility rate in semen diluted of curimba,

being similar to the values found in this study, 88.24 and 90.29%, using 10% methanol and DMSO. The use of cryoprotectants reduces the deleterious effect of the technique on sperm cells, and the positive effect of methanol and DMSO in the variables of motility may be related to its osmolality (HARVEY and KELLEY, 1984) and the rapid intracellular penetration (JAMIESON, 1991).

According MURGAS *et al*. (2014), the high concentration of cryoprotectants can cause significant reduction in sperm quality of fish, but our data show that the use of intra and extracellular cryoprotectants showed no relevant toxicity in any of the combinations tested. MILIORINI *et al*. (2011) found that semen samples of curimba, plus methanol and DMSO, showed motility durations of 43 and 37 seconds respectively, being lower than those found in the present study.

In cryo-toxicity tests, the knowledge of the period of stabilization between the semen and the solutions is necessary and some studies can not assess the real effect of cryoprotectants on the sperm cells (RIBEIRO and GODINHO, 2003). In the present study, this period favored the maintenance of seminal quality of curimba, allowing to frozen samples with guaranteed viability. This period (10 min) is feasible since the procedures that precede the semen freezing fish generally spend little time.

The effect of cryoprotectants can be amplified or inhibited depending on the substances used for such purpose. RIBEIRO and GODINHO (2003) did not consider satisfactory the use of egg yolk in semen cryopreservation of *Leporinus macrocephalus*, and attribute this to the lack of synergy between the cryoprotectants. In cryopreservation trials, MILIORINI *et al*. (2011) also observed that the use of egg yolk, showed some toxicity to the curimba semen and our data also demonstrate a reduction of the rate and duration of sperm motility after thawing, when using this substance as an external cryoprotectant.

MURGAS *et al*. (2007) using methanol and DMSO in semen cryopreservation of curimba, found lower rates and durations of motility, but the combination of intra and extracellular cryoprotectants was not used. The difference in values can be explained by the use of extracellular cryoprotectants, in present study, which promote greater protection to sperm cells.

Freezing and thawing processes can cause changes in spermatozoa protein profiles that may lead to a decreased in semen quality and affect DNA integrity (LI *et al*., 2010), compromising the ability of fertilization. This negative effect on sperm could be observed in the present research, which shows a reduction of almost 50% in the rate of motility compared to fresh semen.

The cryopreservation protocol used in this study showed better motility duration rates when compared to cryopreservation performed by MURGAS *et al*. (2007), who found a duration of motility of 18 seconds. Probably this difference is due to the use of external cryoprotectants combined with DMSO and methanol, which provided greater protection to sperm cells.

Lactose combined with DMSO or methanol may have had a better protection of the mitochondrial membrane system, reducing damage during the process of freezing and thawing. This protection will be able to promote a longer beat the sperm flagellum (MILIORINI, 2006), increasing the chances of fertilization.

The duration of sperm motility in teleost freshwater fish is very short and varies between species (COSSON, 2004; SADIQUL ISLAM and AKHTER, 2011). It is more practical to assume that the rate and duration motility of semen *in natura*, is the one that best fits biology of oocytes, even in controlled conditions than natural habitat. The variation of motility found between different studies may be explained by the individual conditions of the animals and the period of semen collection.

Sperm morphology is also an important factor to be considered, and possibly, the abnormalities found in this work are associated with reduced sperm motility rate. As in the study by MORAES *et al*. (2004), the main changes found in this test were: coiled and fractured tail, and isolated head. These abnormalities undertake the progressive motility of sperm, promoting circular and oscillatory movements that reduce the fertilization rate (KAVAMOTO *et al*., 1999).

The cryoprotectants used in this study protected biochemical characteristics of the

sperm cells, allowing the occurrence of only 25% of morphological changes after thawing. The artificial insemination in curimba uses a high proportion of sperm and oocytes in a controlled environment, so the critical percentage of abnormal sperm oscillate around 50% (MILIORINI *et al*., 2011).

The high frequency of isolated heads may have been due to the breeding season or handling during the experiment, and changes in the midpiece are due to the destabilization of lipoprotein membranes of spermatozoa, when cells are subjected to freezing (MILIORINI *et al*., 2011).

The incidence of fractured tail and isolated head may be related to the process of preparation of slides stained smears for morphological analysis (MORAES *et al*., 2004), and the occurrence of coiled tail was not expected, due to the protective effect of dilutive in the cytoplasmic membrane of the sperm.

CONCLUSIONS

All solutions tested in this study were effective for cryopreservation of curimba semen and not detected differences in the morphological sperm changes. However, lactose stood out as extracellular cryoprotectant, with higher rates and durations for motility of thawed semen, independent of its combination with methanol or DMSO.

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