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Enviroment

Effect of cryoprotectants on the semen quality of Nile Tilapia *Oreochromis niloticus*

Efeito de crioprotetores na qualidade do sêmen de tilápia do Nilo *Oreochromis niloticus*

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ABSTRACT

Samples of Nile Tilapia (*Oreochromis niloticus*) semen were collected with the aim of evaluating the quality of cryopreserved semen using methanol and dimethylacetamide (DMA) as cryoprotectant agents at the concentrations of 7.5% and 10% each. The diluent (base) used in each cryoprotectant consisted of *Beltsville Thawing Solution* (BTS). An examination of the motility factors, robustness, and duration of motility was conducted using sodium carbonate (at 1% solution) for semen activation. The results were higher in methanol regarding robustness, and 7.5% methanol in relation to motility rate and duration of motility. The cryopreservation process increased the number of morphological pathologies, primarily fractured tail, isolated head and folded tail, except for treatment with DMA 7.5%.

Keywords: Tilapia; Cryopreservation; Methanol; Dimethylacetamide

RESUMO

Amostras de sêmen de tilápia (*Oreochromis niloticus*) foram coletadas com o objetivo de avaliar a qualidade do sêmen criopreservado com metanol e dimetilacetamida (DMA) como agentes crioprotetores, nas concentrações de 7,5% e 10% cada. O diluente (base) utilizado em cada crioprotetor consistiu na Solução de Descongelamento de Beltsville (BTS). Foi realizado um exame dos fatores de motilidade, robustez e duração da motilidade, utilizando carbonato de sódio (solução a 1%) para ativação do sêmen. Os resultados foram maiores em metanol quanto a robustez e 7,5% em metanol em relação à taxa de motilidade e duração da motilidade. O processo de criopreservação aumentou o número de patologias morfológicas, basicamente cauda fraturada, cabeça isolada e cauda dobrada, com exceção do tratamento com DMA 7,5%.

Palavras-chave: Tilapia; Criopreservação; Metanol; Dimetilacetamida

1 INTRODUCTION

Cryopreservation is a technique that allows the storage of semen at negative temperatures, the temperature is around -196ºC, thus preserving the stored material. (Jang et al., 2017). The advantage is in the synchronization of the breeders in terms of semen availability; ease of transport between properties; reduction of costs with the maintenance of cores; increase in the useful life of breeders; availability of semen in less favorable periods; expansion of genetic improvement programs; and the possibility of selling semen (Borges et al., 2020). The main advantages of cryopreservation of fish semen are: conservation of genetic variability (germplasm banks); synchronization of breeders in semen availability; ease of transport between properties; reduction of costs with the maintenance of cores; increase in the useful life of breeders; availability of semen in less favorable periods; expansion of genetic improvement programs; and the possibility of selling semen (Navarro et al, 2014; Borges et al., 2020).

Few semen protocols are being developed for the Characidae, Prochilodontidae, Anastomidae, Pimelodidae, Siluridae, Cyprinidae, Salmonidae, Centropomidae, Epinephelidae and Lutjanidae families (Martínez-Páramo et al., 2017). To evaluate the quality of raw and cryopreserved semen of Nile tilapia (Oreochromis niloticus), with different cryoprotectants.

2 MATERIAL AND METHODS

The experiment was carried out at Companhia do Peixe (Cia - fish), located in the rural area of the Western City - Goiás. Approved by the Ethics Committee for Animal Use (CEUA), protocol nº. 66/2017.

Ten male Nile Tilapia breeders were used. The animals were distributed in land tanks in a completely randomized design. The animals were fed an isoprotein diet of 32% PB and isocaloric 3300 kcal ED / kg.

The *puçá* (trawl in funnel shape) was used to catch the animals, which were

contained by a dry cotton towel. The animals were transferred to water tank containing eugenol at the dosage of 65 mg per liter of water. After the animals were anesthetized, each individual ejaculate samples from the males were collected as follows: after capturing, the urogenital papilla was cleaned and dried with paper towels; manual compression of the celomatous wall was performed in the craniocaudal direction; the semen was checked for contamination or activation; those that activated early were discarded; the remaining ejaculates were collected in eppendorf tubes and then wrapped and immersed in ice, protected from light, for further laboratory analysis.

The quality assessment of the semen in natura (and subsequent analyzes) of each male was performed in a 10 µL aliquot of semen deposited on a microscope slide and observed under a light microscope, previously focused on a 40X magnification. Semen was activated by addition of 1% sodium bicarbonate in 1:1 ratio (semen: 1% sodium bicarbonate) to evaluate its quality.

All the ejaculates collected were analyzed afterwards and the rate (0-100%) and duration (seconds) of sperm motility were measured. The motility was measured subjectively under a light microscope. Motility duration was assessed as follows: a timer was triggered at the time of addition of the activating agent and stopped when 10% of the sperm were still moving.

Samples of semen “in natura” were used for morphological analysis. An aliquot of 10 µl of semen in natura was diluted in 990 µl of formaldehyde-citrate solution. Next, a 10 µl fraction of the fixed sample was deposited on a histological blade and covered with cover slip. The examination consisted of observing the morphology of 200 focussed spermatozoa in several fields along the entire slide. The morphological analyzes of the in natura semen were performed under a composite optical microscope with fluorescent episcopic lighting (Nikon, model OPTIPHOT-2), at the UnB Aquaculture Laboratory.

The semen in natura was evenly distributed among four sterile eppendorf tubes. A slow and gradual dilution of the aliquots was performed by four cryoprotective

solutions, in the proportion of one part of semen to nine parts of solution. The solutions were composed of two cryoprotectants, methanol and dimethylacetamide (DMA), in two concentrations (volume: v - v: v): 7.5% and 10%. The diluent solution (base) for each cryoprotectant consisted of BTS-Beltsville Thawing Solution (79.90 g of glucose, 12.71 g of sodium citrate, 2.65 g of ethylenediaminetetraacetic acid, 2.65 g of sodium hydrogen carbonate, 159 g of potassium chloride and 0.50 g of gentamicin sulfate).

All cryoprotectant solutions were prepared one hour in advance of the dilution for the stabilization of the cryoprotectants at the end of the exothermic reactions harmful to sperm cells.

After dilution by the cryoprotectant solutions, the samples were filled in 0.5 ml vials (two vials per treatment), which, in turn, were sealed with polyvinyl alcohol. The vanes were packed in racks and placed in liquid nitrogen (Taylor-Wharton, model CP 300, dry shipper type) for cooling. After 24 hours, the racks were transferred to a storage vessel (Cryometal, model DS-18) and frozen, remaining submerged in liquid nitrogen.

The thawing occurred in the Aquaculture Laboratory of the University of Brasilia, the vats being thawed individually by immersion in water (water bath) at 40 ° C for twelve seconds and shaken throughout the procedure. They were then wiped with a paper towel, the end with polyvinyl alcohol having been discarded and the semen deposited in a sterile eppendorf tube.

A 10 µl aliquot of thawed semen was deposited on a histological slide previously focused on an optical microscope under 100 dioptre increase. After confirmation of absence of activation, the sample was activated by homogenization with 40 µl of distilled water or 1% NaHCO 3 solution. Two scales of semen motile titration were compared, one quantitative and another qualitative.

By the quantitative scale, the sperm motility was estimated as a function of the average percentage of mobile spermatozoa observed in three fields. The qualitative scale was attributed to vigor of motility divided into categories from 0 to 5, according

to their performance. The duration (in seconds) of sperm motility was also estimated from the homogenization with the activator until only 10% of the spermatozoa in the field were mobile. The following statistical analyzes were used: ANOVA and Tukey mean test, considering 1% and 5% probability.

3 RESULTS

The results obtained in the experiment are presented in the tables below, which distinguish the different treatments, basically two cryoprotectants, methanol and DMA - Dimethylacetamide, both with two concentrations, 7.5% and 10%.

Table 1 – Mean of motility rate (%), vigor (0-5) and duration (seconds) of motile spermatozoa in natura and after thawing

Sample		Motility Rate (%)	Stamina (0 - 5)	Motility Duration (sec)
In natura		70,00±16,58	3,43±0,79	231,86±76,48
Treatments		Motility Rate (%)	Stamina (0 - 5)	Motility Duration (sec)
Methanol	7,5%	30,00±31,89^{a}	3,14±0,38^{a}	397,14±363,2^{a}
	10,0%	25,00±18,93^{ab}	3,00±0,58^{a}	228,86±128,93^{ab}
DMA	7,5%	4,43±2,88^{b*}	1,71±0,49^{b**}	100,29±28,74^{b*}
	10,0%	3,86±1,21^{b*}	1,57±0,53^{b**}	106,86±41,74^{b*}

In a column, values with the same letter do not differ from each other; letters followed by an asterisk (*) mean P <0.05); Letters followed by asterisks (**) mean P <0.01

It was observed that the mean value of the motility rate of the treatments with methanol at 7.5% was significantly higher than the treatments with DMA, and there were no differences between the other treatments between them (table 1).

As for sperm vigor, the mean values (Table 1) show that the two methanol treatments presented higher results than those with DMA, with no difference between the two concentrations.

Regarding the duration of motility, the mean values, shown in Table 1, show that treatment with 7.5% methanol presents a significantly higher result than DMA

cryopreserved, with no significant difference between methanol concentrations and between methanol 10% and treatments with DMA.

Regarding the morphology of spermatozoa, the following aspects were examined in relation to the in natura sample and the four treatments: normal, macrocephaly, microcephaly, degenerate head, degenerate intermediate part, fractured tail, strongly coiled tail, degenerate tail, isolated head, proximal drop, distal droplet and folded tail, the results of which are shown in table 2.

When related to the *in natura* sample, the cryopreserved spermatozoa with 7.5% methanol presented a superior quantitative of individuals with fractured tail and folded tail; with 10% methanol, a lower number of normal and larger with fractured tail occurred; with DMA 10%, the number of normals was lower, with the isolated head and fractured tail being higher (Table 2).

It is observed that the variations between the *in natura* sample and the treatments show negative results of these. Among the treatments, there were no differences.

Table 2 – Means of morphological characteristics of Nile tilapia spermatozoa in natura

	Normal	**Macrocefalia**	**Microcefalia**	**Degenerate head**	**Degenerate intermediate part**	**Fractured tail**
In natura	74,00±17,49	5,28±4,70	0,07±0,19	1,50±1,15	0,42±0,61	1,64±1,21
	heavily curled tail	degenerate tail	normal isolated head	proximal drop	distal drop	bent tail
In natura	12,92±13,71	0,07±0,19	2,57±1,62	0,21±0,39	0,21±0,39	1,07±0,93

Table 3 – Means of morphological characteristics of Nile tilapia spermatozoa after freezing

		Normal	Macrocefalia	Microcefalia	Degenerate head	Degenerate intermediate part	fractured tail
Metanol	7,5%	57,07±9,71	8,14±4,24	0,14±0,24	2,21±1,78	2,21±2,14	7,50±2,90
	10%	54,57±4,86	7,07±4,63	0,07±0,19	2,50±1,68	3,07±2,94	6,64±3,61
DMA	7,5%	59,28±10,69	8,14±4,84	0,21±0,57	1,29±0,64	2,00±3,38	6,29±3,24
	10%	53,28±11,52	7,42±5,72	0,00	1,07±1,06	1,21±1,95	8,07±3,61
		heavily curled tail	degenerate tail	normal isolated head	proximal drop	distal drop	bent tail
Metanol	7,5%	12,14±7,34	0,14±0,24	6,07±2,51	0,14±0,24	0,21±0,39	4,00±0,93
	10%	15,14±4,55	0,29±0,57	7,21±2,08	0,07±0,19	0,00	3,36±3,16
DMA	7,5%	9,57±5,63	0,21±0,39	9,00±4,88	0,07±0,19	0,00	3,93±1,60
	10%	13,21±8,36	0,14±0,24	12,00±9,97	0,14±0,24	0,00	3,43±1,10

Figure 1 – Images showing spermatozoa with abnormalities indicated by arrows. (A) Macrocephaly; (B) Degenerate head; (C) degenerate intermediary piece; (D) Fractured tail; (E) Strongly curled tail; (F) degenerate tail; (G) Isolated head and (H) Folded tail

(continued)

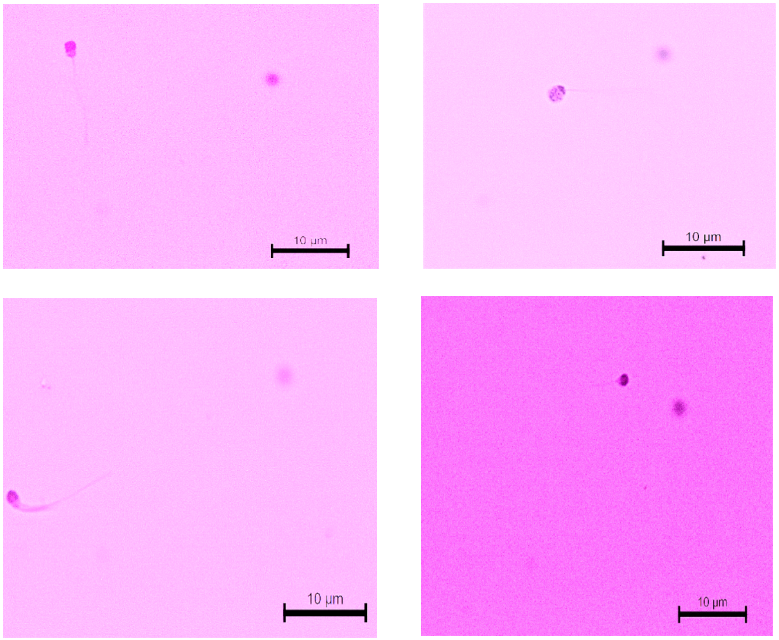
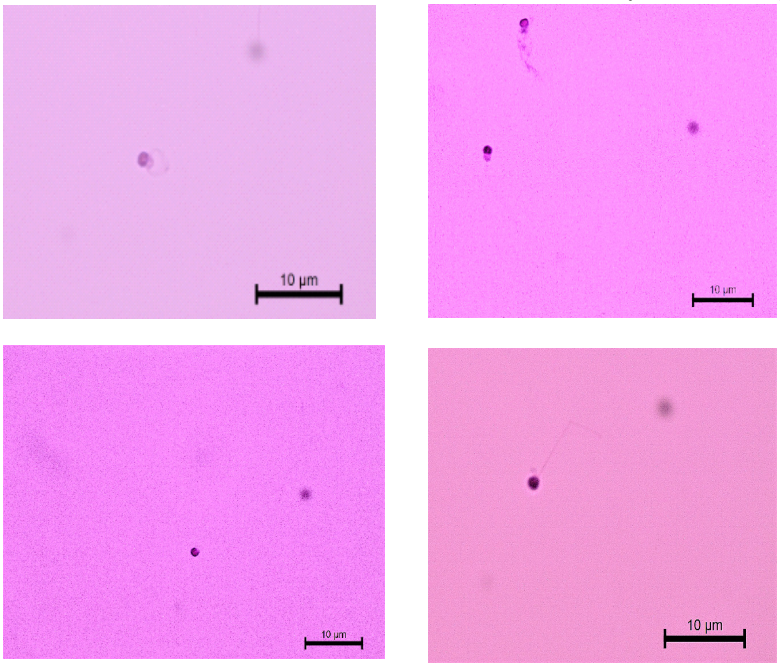


Figure 1 – Images showing spermatozoa with abnormalities indicated by arrows. (A) Macrocephaly; (B) Degenerate head; (C) degenerate intermediary piece; (D) Fractured tail; (E) Strongly curled tail; (F) degenerate tail; (G) Isolated head and (H) Folded tai

(conclusion)



4 DISCUSSION

The values of motility rate after freezing with 7.5% methanol as cryopreservant were higher and more significant than those obtained with DMA, at both 7.5% and 10%. This result is correspondent with those of several studies, the hypotheses for such behavior are presented: Godinho et al. (2003), justifies that the low motility with the use of DMA is due to its high toxicity to Nile tilapia. Godinho et al. (2003). However, when evaluating Nile Tilapia motility rates using cryopreservants methanol and dimethyl sulphoxide (DMSO), it reached different conclusions and did not achieve significant results.

Harvey (1983) conducted a study on the freezing of sarothedoron mossambicus semen by comparing several cryoprotectants, including methanol and DMSO, both with a 15% percentage, diluted in skimmed milk powder and chicken egg yolk, obtained

higher values of methanol for the methanol diluted in milk powder, in comparison with the other cryoprotectants, concluding, therefore, that methanol is also the most suitable for the species.

Ohta et al. (2001), in the work on freezing of bitterling japanese semen, comparing 10% methanol diluted in bovine serum, and DMA, concluded that methanol was the most indicated in cryopreservation of semen of this species, obtaining 40% of motility with the use of methanol and 30% with the use of DMA.

Despite the values obtained in the present work, the DMA may be indicated for the cryopreservation of other species. In a study using African catfish semen, Horvath and Urbanyi (2000) used the cryoprotectants methanol, ethylene glycol, propylene glycol and glycerol diluted to 5.10 and 15% in solution of fructose monohydrate, dimethylsufoxide (DMSO) and dimethylacetamide (DMA) at the same diluent. The results suggested DMSO as the most effective for the species, but DMA can also be used as a cryoprotectant for the species and causes less damage to cells than DMSO, with similar fertility rates. The study indicated that methanol causes less damage to cells, but is less effective than DMSO. Ethylene glycol was not considered to be indicated for cryopreservation.

Researching Arctic trout semen, Richardson and McNiven (2000) used DMA, DMSO and glycerol, having concluded that DMSO is more effective for the species. With the DMA obtained superior motility values with 2 minutes after the activation, however, the values do not have significant difference after this period; with glycerol obtained significantly lower results than the other cryoprotectants.

Methanol achieved superior results of percentage motility over both of the DMA concentrations. Values found in the present study are in disagreement and above those obtained by Menezez et al. (2008), working with tambaqui (Colossoma macropomum), and using DMA, DMSO, ethylene glycol, propylene glycol and methanol as cryoprotectants, obtained results below 5% for DMA and methanol, between 5% and 10% for DMSO and 20% to 25% for ethylene glycol and propylene glycol.

The vigor with the methanol treatments presented a significant difference in relation to that of the DMA. Another study, such as Streit *et al*. (2009), with DMSO cryopreserved pacu semen also presented a reduction in the vigor of the samples after thawing and observed that vigor and motility factors are closely related and may result from morphological pathologies.

Some pathologies classified as secondary are associated with the process of semen manipulation during its collection, among them are macrocephaly, microcephaly and broken, degenarated or coiled flagellum. The primary pathologies, such as isolated head, folded flagellum, and proximal and distal droplets are caused by consanguinity, environmental stress, diseases and dietary restrictions. (Herman and Doak 1994 apud Melo-Maciel et al., 2012).

In this study the semen in natura presented 26% of morphological pathologies. The pathologies that yeiled the highest occurrence after freezing were: fractured tail (secondary), isolated head and folded tail (both primary).

Sperm structures are important for the reproductive performance of fish (head, middle part and tail) (Kavamoto et al., 1999, Navarro et al., 2021). Milliorini et al., (2011) sperm abnormalities in external fertilization fish, as well as tilapia, should be around 50%.

5 CONCLUSIONS

Cryopreservation of Nile Tilapia semen with methanol in concentrations of 7.5% showed better results for semen quality. This work may guide further research with the aim of economically valuing the activity, as well as increasing the number of fingerlings destined for aquaculture.

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