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Semen quality of Curimba (*Prochilodus lineatus*) cryopreserved with vitamins

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ABSTRACT. The present study examined the effect of adding the antioxidants vitamins C and E on the quality of semen cryopreserved of curimba (*Prochilodus lineatus*). Semen samples from nine breeding males were collected for analysis of rate (%) and duration (s) of sperm motility. The sperm pool was diluted into three cryoprotective solutions: Solution A: 5 gr. Beltsville Thawing Solution (BTS) added with 5 mL dimethyl sulfoxide (DMSO) and distilled water to 100 mL; Solution B: Solution A + 0.0001 mg vitamin E; Solution C: Solution A + 0.0001 mg vitamin C. The vitamins C and E were not toxic to the semen of curimba. The sperm motility did not present any significant difference. However, the semen cryopreserved with vitamin C had a longer duration of the motility after thawing. Therefore, the vitamin C is recommended for the cryopreservation of the semen of curimba.

Keywords: sperm motility, fish, reproduction, cryoprotective solution.

Qualidade de sêmen de Curimba (*Prochilodus lineatus*) criopreservados com vitaminas

RESUMO. O presente estudo avaliou o efeito da adição de vitaminas antioxidante C e E sobre a qualidade do sêmen criopreservado de curimba (*Prochilodus lineatus*). As amostras de sêmen de nove machos reprodutores foram coletadas para análise da taxa (%) e duração (s) da motilidade espermática. O pool de esperma foi diluída em três soluções crioprotetores: Solução A: 5 gr. de solução de descongelamento Beltsville (BTS) adicionaram-se 5 mL de dimetilsulfóxido (DMSO) e água destilada para 100 mL; Solução B: Solução A + 0,0001 mg de vitamina E; Solução C: Solução A + 0,0001 mg vitamina C. A vitamina C e E não foram tóxicos para o sêmen de curimba. A motilidade espermática não apresentou diferença significativa. No entanto, o sêmen criopreservado com a vitamina C teve uma duração mais longa da motilidade pós-descongelamento. Portanto, a vitamina C é recomendada para a criopreservação do sêmen de curimba.

Palavras-chave: motilidade espermática, peixe, reprodução, solução crioprotetora.

Introduction

Fish sperm banks consist of files of genetic material cryopreserved whose use is essential in fish farmings and in conservation programs for endangered species (RIBEIRO; GODINHO, 2003). Among the benefits, this technique eliminates the reproductive asynchrony between males and females, assists the conservation of genetic variability in domesticated populations, and eases the establishment of breeding programs (CAROLSFELD et al., 2003; RIBEIRO; GODINHO, 2003; MENEZES et al., 2008).

The successful cryopreservation of semen depends on the use of suitable cryoprotective solutions at ideal concentrations and of antioxidants (PAULA et al., 2012; NAVARRO et al., 2009). The motility rates after thawing and the fertilization tests are the most appropriate criteria used to evaluate the success of the cryopreservation (MENEZES et al., 2008; NAVARRO et al., 2009).

The sperm cryopreservation is an important technique for aquaculture and has produced significant contributions for long-term preservation of semen for artificial reproduction of several fish species (MURGAS et al., 2007; VIVEIROS et al., 2009), such dourado (*Salminus brasiliensis*) (CAROLSFELD et al., 2003), matrinxã (*Brycon cephalus*) (SILVEIRA et al., 2006), Nile tilapia (*Oreochromis niloticus*) (GODINHO et al., 2003), tambaqui (*Colossoma macropomum*) (MENEZES et al., 2008) and curimba (*P.lineatus*) (FELIZARDO et al., 2010).

The curimba, *Prochilodus lineatus*, is a migratory fish, native of South America (NAVARRO et al., 2007; VIVEIROS; GODINHO, 2008). This medium sized fish plays a key role in the ecosystem, as well as in commercial and subsistence fishing in Southeastern Brazil, with a high productivity in fish farmings (MADUENHO; MARTINEZ, 2008). By being a spawning species, the curimba has its reproductive cycle affected by the urbanization, pollution, deforestation, overfishing, and dam construction (VIVEIROS et al., 2009).

The sperm cryopreservation is an alternative to promote the preservation of stocks of curimba. The fish sperm can be cryopreserved at temperatures below 0°C without any deterioration (CARNEIRO, 2007). The cryopreserved sperm can be used in captive breeding, increasing the production of larvae and the creation of a sperm bank, which ensures the genetic diversity and the reproductive success (VIVEIROS et al., 2009).

Studies on sperm cryopreservation in Brazil have focused on rheophilic fish species, such as dourado, *Salminus brasilienses* (CAROLSFELD et al., 2003); piracanjuba, *B. orbignyanus* (CAROLSFELD et al., 2003; MARIA et al., 2006); matrinxã, *Brycon cephalus* (SILVEIRA et al., 2006), and also curimba, *P. lineatus* (MURGAS et al., 2007). However, reports on the association between antioxidant and cryoprotective solutions in the process of fish sperm cryopreservation are scarce.

For a successful cryopreservation, the composition of the cryoprotective or dilution solution is very important to achieve an adequate survival rate. With this purpose, dozens of dilution solutions have already been developed, and the addition of different cryoprotective substances to the dilutor has been tested, among them the antioxidants (MILIORINI et al., 2011).

The addition of antioxidants to dilution solutions for semen freezing has been used in several mammalian species, aiming to minimize or reverse the damaging effects caused by reactive metabolites of oxygen (O_2^-, OH, H_2O_2) to the sperm cell (NAVARRO et al., 2009, 2012).

The spermatozoon has a intracellular antioxidant defense system against the ROS – reactive oxygen species, which consists basically of enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase and reductase, and non-enzymatic antioxidants, such as ascorbic acid and α-tocopherol (THUWANUT et al., 2008). Extracellularly is protected by seminal plasma that contains several reducers of RSO, enzymatic or not, contributing to a powerful antioxidant activity. These antioxidants include the ascorbic acid, uric acid, albumin and other proteins, catalase, SOD, glutathione and other thiols, taurine, hypotaurine, and vitamin E (OVERVELD et al., 2000).

The vitamin E (α-tocopherol and derivatives), predominant animal fat-soluble antioxidant, protects the cells against oxygen radicals, *in vivo* and *in vitro*, and is believed to be the primary inhibitor of free radicals, found at low amounts in cell membranes of mammalian and in seminal plasma, protecting the cells from DNA damage (SIKKA, 2004) and oxidative stress (MONTEIRO et al., 2009; NAVARRO et al., 2010).

The vitamin C also acts synergistically with the vitamin E, through the generation of tocopherol from tocopheroxyl radicals, product of the interaction of tocopherol and free radical oxygen. In this way, the vitamins C and E act together, by protecting the lipid peroxidation, reducing the production of ROS induced by H_2O_2 and protecting the sperm against DNA damage (GUERRA et al., 2004).

In this way, the present study aimed at evaluating the effect of vitamins C and E on the quality of sperm of curimba (*P. lineatus*) during the cryopreservation process.

Material and methods

The experiment was conducted in the Fish Farming Station of the Federal University of Lavras (UFLA), from January 1st to 31st, 2010.

The sperm was collected from nine males of *P. lineatus* with weight (310 ± 48 g) and length (29.4 ± 1.74 cm), respectively. Fish were captured from cement tanks of the Station using a seining net, being selected the animals eligible to receive hormonal induction according to the reproductive characteristics: erythematous color, and edematous appearance of the urogenital papilla, and release of sperm in response to manual massage on the abdominal wall.

The selected individuals were taken to the laboratory and individually placed in treatment tanks of 100 L. Fish fasted for 24 hours before hormone injection.

Fish received two intramuscular injections of crude extract of carp pituitary (EBHC), being the previous dose of 0.4 mg kg^{-1}, and the definitive dose of 4.0 mg kg^{-1}, in a time interval of approximately 12 hours between the injections. The sperm was collected from nine to 10 hours after the second injection.

The temperature was measured daily. The temperature was measured by a digital device Brand Bernauer F-1002, Blumenau, Santa Catarina State, Brazil.

For the individual collection of the ejaculate, the fish was captured with a dip net, and restrained with a dry cotton towel. The urogenital papilla was cleaned up, and dried with paper towel to prevent the prior activation of the sperm, before contact with the water, feces or urine of the animal. Gentle manual compressions were performed on the abdominal wall, in the cranio-caudal direction. After ejaculation, the sperm was collected into sterile test tubes. An aliquot with 10 μl of fresh sperm was collected from each animal and placed on a histological glass slide, homogenized with 40 μl distilled water at a ratio of 1:4 (sperm:water). The sperm motility was examined under light microscopy, with a magnification of 100 diopters, and estimated in average percentage of motile sperm observed in three fields. The duration of the sperm motility was estimated from the homogenization with distilled water until only 10% of sperm in the field was found mobile. It was only considered the samples with sperm motility rate of 100% and without prior activation of motility, according to (MILIORINI et al., 2011).

To accomplish the cryopreservation process, a sperm pool was obtained from the nine males selected. The pool was diluted into three different cryoprotective solutions, at a ratio 1:4 (sperm:cryoprotective solution) and distributed into three straws for each treatment, totaling nine straws. The cryoprotective solutions were first prepared, in order to stabilize until being used, and were composed as follows:

The BTS is a diluter developed and recommended for conservation of boar sperm.

Solution A:

5 grams Beltsville Thawing Solution (BTS) added with 5 mL dimethyl sulfoxide (DMSO) and distilled water to 100 mL

Solution B:

5 grams BTS added with 5 mL DMSO and 0.0001 mg vitamin E (tocoferol acetate) and distilled water to 100 mL.

Solution C:

5 grams BTS added with 5 mL DMSO and 0.0001 mg vitamin C (ascorbic acid) and distilled water to 100 mL.

The solution A, without any antioxidant, was used as a control solution.

The sperm diluted in the different cryoprotective solutions was evaluated as for the rate (%) and duration (s) of sperm motility, in order to check the toxicity of the cryoprotective before freezing, using the same method used to evaluate the fresh sperm.

Then, the samples were stored in straws with 0.5 of capacity, generating a total of three straws/sperm sample for each treatment. The straws were labeled and placed in a liquid nitrogen vapor dry shipper (Taylor-Wharton, model CP 300) and kept in vertical position. After 24 hours, they were transferred to a liquid nitrogen canister (Cryometal, model DS-18) at a stable temperature of -196ºC. After three days of freezing, the sperm samples were thawed.

The thawing involved the withdrawal of the straws from the canister and immersion in a preheated water bath, at 60°C for eight seconds. Then, the straws were dried with paper towel, and had their ends cut with scissors to remove the sperm, which was placed on a Petri dish. Afterwards, it was evaluated the rate (%) and duration (s) of sperm motility, according to the method used for the fresh sperm (MILIORINI et al., 2011).

The analysis of rate and duration of sperm motility was performed in a completely randomized design with three treatments and two replications. The measurements of rate and duration of motility of the fresh sperm, pre-freezing and post-thawing, were compared by a Student-Newman-Keuls test at 5% probability, using the software SAS.

Results and discussion

The water temperature of the tanks remained at 27 ± 1°C, during the study period. No significant difference $p > 0.05$ was detected for the motility rate and duration of motility of the diluted sperm in the pre-freezing with the different antioxidants used (Table 1). All the treatments presented 100% of motility rate, and duration of sperm motility above 60 seconds. Other authors observed a significant improve of total motility with addition of α-tocopherol and ascorbic acid in European sea bass (MARTINEZ-PÁRAMO et al., 2012). However, Leung (1991) reported that at high concentrations, the cryoprotective solutions with vitamins can be toxic to sperm and may reduce the sperm viability. Some researchers have found an increased motility rate of the sperm of Russian sturgeon (Acipenser gueldenstaedti) protected with 10 mM ascorbic acid, as well as a reduction of chromosomal aberrations in developing embryos (MIRZOYAN et al., 2006).

The importance of the duration of sperm motility is in the time required by the sperm to penetrate the oocyte micropyle and for fertilization to occur. In most teleosts, the time of opening of the micropyle is around 60 seconds (ANDRADE; YASUI, 2003; RICARDO et al., 1996). In the present experiment, before freezing, all the treatments attained the duration of sperm motility above 60 seconds. Another study of Miliorini et al. (2011) reported duration between 24 and 88 seconds for the sperm of curimba.

Table 1. Rate (%) and duration (s) of sperm motility of fresh semen, diluted in pre-freeze and post freeze curimba *Prochilodus lineatus.*

Fresh semen and before freezing	Motility rate (%)	Duration of motility (s)
Control (no antioxidant)	100 [a]	68 ± 1.71 [a]
Semen Vitamin E	100 [a]	67 ± 0.69 [a]
Semen Vitamin C	100 [a]	65 ± 0.70 [a]
Fresh semen	100 [a]	72 ± 1.34 [a]
C.V.	-	4,32
Semen post freeze	Motility rate (%)	Duration of motility (s)
Control (no antioxidant)	45.0 ± 7.0 [a]	52,5 ± 0.70 [a]
Semen Vitamin E	60.0 ± 20.0 [a]	27,0 ± 9.78 [b]
Semen Vitamin C	75.0 ± 10.0 [a]	54,5 ± 2.12 [a]
C.V.	25	34

Averages in the same column with different superscript are significantly different according SNK test ($p < 0.05$). Average ± EPM, CV - coefficient of variation.

The spermatozoa of fish are morphologically subdivided into head, middle piece, and tail (COWARD et al., 2002). The acrosome is a structure absent in fish sperm, being compensated by the micropyle, a hole in the oocyte chorium where the sperm cell penetrates (GANECO; NAKAGHI, 2003).

After thawing, the sperm did not present any significant difference $p > 0.05$ for the rate of sperm motility (Table 1). A significant difference was verified for the duration of sperm motility for the treatments cryopreserved without antioxidant, and with vitamin C, in relation to the sperm cryopreserved with vitamin E. This increase promoted by the vitamin C, associated with a duration of motility of 54.5 seconds, can favor a good fertilization rate. However, the significant reduction ($p < 0.05$) in the motility duration, after thawing, of the sperm cryopreserved with vitamin E may lead to low fertilization. Although the sperm motility had not been different after thawing between the antioxidants used in the cryopreservation, the use of the vitamins C and E have promoted an increase of 30 and 15% in the motility rate, respectively, in relation to the control. This increase promoted by the vitamin C, associated with a duration of motility of 54.5 seconds, can favor a good fertilization rate. However, the significant reduction ($p < 0.05$) in the motility duration, after thawing, of the sperm cryopreserved with vitamin E may lead to low fertilization. The duration of sperm motility was much lower than the average time of opening of the micropyle, 27 seconds, and in this way it can reduce the sperm penetration ability. The effects of the vitamin E can vary depending on the dose, once according to the amount of hydroxyl radicals to be inactivated the vitamin E can have antioxidant effect or stimulate the oxidation (NAVARRO et al., 2009). CABRITA et al. (2011) reported that the addition of antioxidants (1 - 10 mM vitamin C and 0.1 - 0.5 mM vitamin E) did not led to a significant increase in motility and viability in post-thawed sperm of *Sparus aurata* and *Dicentrarchus labrax.*

This is a pioneering study on the use of antioxidants for the cryopreservation of sperm of curimba, which instigates the accomplishment of future experiments to increase the knowledge on this issue. The cryopreservation process reduced the motility rate of post-thawed sperm, compared with the sperm before freezing. Murgas et al. (2007), assessing cryoprotective solutions to preserve sperm of curimbatá, also observed that the cryopreservation process has decreased the sperm motility rate, after thawing. The processes of freezing and thawing cause sperm mortality, besides damaging the cell structures, which can disable them for fertilization (MARTINEZ; EKWALL, 1998), emphasizing thus the importance of adding antioxidant substances to cryoprotective solutions.

Conclusion

The use of vitamins C and E had no effect on the quality of sperm of curimba before the freezing. The addition of the antioxidant vitamin C at 0.0001 mg is recommended for the cryopreservation of curimba sperm by promoting, after thawing, greater increases in motility rate, of 30 and 15%, and in duration, of 2.0 and 25.5 s, respectively, in relation to the control and treatment with vitamin E.

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