Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1806-2563
ISSN on-line: 1807-8664
Doi: 10.4025/actascitechnol.v36i3.20076

Cryopreservation of semen of Thailand tilapia (*Oreochromis* spp.) fed diet with different oil sources

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ABSTRACT. This study evaluated the quality of sperm cryopreserved with dimethylsulfoxide (DMSO) or methanol, of Thailand tilapia (*Oreochromis* spp.) fed diet with different oil sources. eighty Thailand tilapia fed different oil sources (T1: linseed oil, T2: soybean oil, T3: fish oil, T4: corn oil) and were analyzed as for motility rate, and duration of motility in 'fresh sperm' and after cryopreservation. No significant difference was found for the final weight, total length, rate and duration of motility of fresh sperm in the different treatments. Significant differences were observed between fish, corn and linseed oil treatments on the rate of motility relative to other different oil sources with the cryoprotectant DMSO prior to freezing. After thawing, fish fed diet supplemented with fish oil presented a significantly higher rate and duration of sperm motility. Nevertheless, for better reproductive parameters after freezing, it is recommended the use of fish oil associated with cryoprotectant methanol.

Keywords: freeze, cryoprotectant, DMSO, methanol.

Criopreservação de sêmen de tilápia do Nilo, alimentada com diferentes fontes de óleo

RESUMO. Este estudo avaliou a qualidade do esperma criopreservados com dimetilsulfóxido (DMSO) ou metanol, da tilápia Tailandesa (*Oreochromis* spp.), alimentada com diferentes fontes de óleo. Foram utilizadas 80 tilápias Tailandesas alimentadas com quatro fontes de óleo (T1: óleo de linhaça, T2: óleo de soja, T3: óleo de peixe, T4: óleo de milho). Foram analisadas a taxa de motilidade e a duração de motilidade do sêmen *in natura* após a criopreservação. Nenhuma diferença significativa foi encontrada para o peso final, comprimento total, taxa e duração da motilidade do esperma fresco nos diferentes tratamentos. Foram observadas diferenças significativas para óleo de milho, de peixe, de linhaça sobre a taxa de motilidade, utilizando o crioprotector DMSO antes da congelação. Após a descongelação, o peixe submetido a uma dieta suplementada com óleo de peixe apresentava uma taxa significativamente mais elevada e duração da motilidade dos espermatozoides. No entanto, para os melhores parâmetros de reprodução após a congelação, é recomendado o uso de óleo de peixe associado com crioprotetor metanol.

Palavras-chave: congelamento, crioprotetores, DMSO, metanol.

Introduction

A cost-effective aquaculture depends primarily on a reliable supply of fertile eggs and fingerlings, which can be produced with breeding stock, kept under suitable feeding conditions. However, studies on nutrition of breeding animals are lacking and are relatively expensive, due to the need of large inner and outer facilities to keep large groups of adult fish, besides the high costs to conduct long-term feeding experiments (IZQUIERDO et al., 2001, SADIQUL ISLAM; AKHTERTALE, 2011).

Sperm cryopreservation is a simple technique used for species preservation, biodiversity conservation, protection of valuable strains, besides being a tool that eases the animal reproduction (ORFÃO et al., 2011). During the cryopreservation, some factors may change the physiological status of sperm. The cryopreservation allows the sperm storage at −196°C for unlimited time, preserving its original conditions (CARNEIRO, 2007; VIVEIROS; GODINHO, 2009; DZIEWULSKA et al., 2011, VIVEIROS et al., 2011).

The main factors are: cryoprotectant used and its concentration, diluent composition, ratio of diluted semen, equilibration time, freezing and thawing rates, semen characteristics, morphology and physiology of sperm of the fish species studied. These factors and the complexity of the cryopreservation process usually require developing species-specific protocol for cryopreservation (DZIEWULSKA et al., 2011).

This process of cryopreservation requires the addition of diluent media with osmotic and nutritional

characteristics suitable for sperm. The dilutor BTS (Beltsville Thawing Solution®), used in swine sperm, has been successfully used in cryopreserving fish sperm (FELIZARDO et al., 2010). However, along with the dilutor, it is necessary to add cryoprotectants to prevent cryoinjuries caused during the process (MARIA et al., 2006). The cryoprotectants DMSO (D*imethyl* sulfoxide) and methanol have shown satisfactory results for *Prochilodus lineatus* (FELIZARDO et al., 2010; MURGAS et al., 2007) and several native species (VIVEIROS et al., 2009; PAULA et al., 2012), but not for *Colossoma macropomum* (MENEZES et al., 2008). According to Godinho et al. (2003), Nile tilapia has obtained the best result with the use of methanol.

The influence of diet on the reproductive performance of fish allows the selection of ingredients at levels suitable to animal metabolism (NAVARRO et al., 2010). Recent studies have been accomplished focusing the improvement of the potential of fish farming, but few studies have associated nutrition with reproductive parameters (EL-SAYED et al., 2005; BOMBARDELLI et al., 2010; NAVARRO et al., 2014).

Reports are lacking on the influence of diet on the semen quality and cryopreservation of sperm of tilapia. Given this, we evaluated the reproductive performance and viability of cryopreserved sperm of Thailand tilapia (*Oreochromis* spp.) fed diets with different oil sources.

Material and methods

The experiment was performed with adult Thailand tilapia (*Oreochromis* spp) with initial weight of (167 ± 0.88 g) gat the Fish Farming Station of the Federal University of Lavras, Lavras, Minas Gerais State, Brazil, during 2 months.

In the experiment was used 80 male tilapia, 20 males for each treatment. The experiment was a completely randomized design with eight treatments in a 4 x 2 factorial arrangement, with four repeat, which used a 32% CP diet isoproteic and isoenergetic 3,300 kcal DE kg-1 implemented with four oil sources T1: linseed oil (OL), T2: soybean oil (OS), T3: fish oil (OF), and T4: corn oil (OC) (Table 1) and two semen cryoprotectants (DMSO and methanol), on two occasions, before freezing and after thawing. The experiment was divided into two phases. Phases one: Feeding Thai tilapia with different sources of oil. In phase two: evaluation of semen from tilapias of phase 1, and verification of using two cryoprotectants (DMSO and Methanol) before freezing and after freezing.

Diets were pelleted and portions corresponding to 5 percent of body weight were offered three times a day (8:00 am, 1:00 and 6:00 pm). Portion size was adjusted every 15 day to accompany fish growth. Fifteen percent of the fish were collected in 3 cm-mesh nets and measured with a caliper and precision scale. A 12:12h light/dark cycle was adopted. Temperature was measured twice a day (7:00 am and 5:00 pm) and pH, dissolved oxygen, measured by a digital oximeter (YSI Bernauer, Company Bernauer, Blumenau, Brazil) and ammonia every 7 day. The pH and temperature were measured by digital a multi-parameter measurer pH by pHmeter (Bernauer, Company Bernauer, F-1002, Blumenau, Brazil).

Table 1. Chemical and percentage composition of the experimental diets.

Ingredients (%)	Diet - Different oil sources			
	Soybean (OS)	Linseed (OL)	Fish (OF)	Corn (OC)
Soybean meal (%)	60.00	60.00	60.00	60.00
Wheat meal (%)	21.16	21.16	21.16	21.16
Corn (%)	9.80	9.80	9.80	9.80
Soybean oil (%)	6.75	-	-	-
Linseed oil (%)	-	6.75	-	-
Fish oil (%)	-	-	6.75	-
Corn oil (%)	-	-	-	6.75
Ducalcium phosphate	0.10	0.10	0.10	0.10
Vitamin and mineral premix (%)[3]	0.60	0.60	0.60	0.60
Limestone (%)	0.62	0.62	0.62	0.62
BHT (Antioxidant)	0.02	0.02	0.02	0.02
Methionine (%)	0.55	0.55	0.55	0.55
Salt (%)	0.40	0.40	0.40	0.40
Nutritional Levels				
Crude Protein (%)[1]	31.98	31.98	31.98	31.98
Digestible energy (kcal kg^{-1})[2]	3299.02	3299.02	3299.02	3299.02
Ether extract (%)[1]	8.01	8.01	8.01	8.01
Crude Fiber (%)[1]	5.71	5.71	5.71	5.71
Calcium, (%)	0.48	0.48	0.48	0.48
Total phosphorus (%)	0.59	0.59	0.59	0.59

[1]Determined at the Animal Nutrition Laboratory (DZO, UFLA). [2]Based on reference values established by Furuya (2010). [3]Commercial vitamin premix (5 kg ton^{-1}), with 1 kg diet containing: 1,200,000 IU vitamin A; 200,000 IU vitamin D_3; 2,400 mg vitamin K_3; 4,800 mg vitamin B_1; 4,800 mg vitamin B_2, 4,000 mg vitamin B_6, 4,800 mg vitamin B_{12}; 1,200 mg folic acid; 12,000mg Ca pantothenate; 48,000 mg vitamin C; 48 mg biotin; 108,000 mg choline chloride; 24,000 mg niacin; Vit. C, 48.000 mg. Commercial mineral premix, with 1 kg diet containing: 50,000 mg Fe; 3,000 mg Cu; 20,000 mg Mn; 3,000 mg Zn; 100 mg I; 10 mg Co; and 100 mg Se.

Animals and facilities

In the first phase of the experiment, the four treatment groups fed different sources of oil in a completely randomized experimental design with four treatments, and four replications, similar to the methodology used by Navarro et al. (2006) and Parra et al. (2008). Fish were subjected to an acclimation period of 5 days.

Fish handling and sperm collection

In phase two, after the feeding period, the animals were taken to 1000 liters-aquaria for later semen collection. For the individual collection of ejaculate, it was used a dip net for capturing the animals that were restrained with a dry cotton towel. They were blindfold and the urogenital papilla was cleaned and dried with a paper towel. Gentle hand pressures were

performed on the abdominal wall, in the cranial-caudal direction. Semen was checked as for contamination or activation. When early activated, the semen was disposed. The ejaculate was collected into sterile test-tubes, immersed in ice, protected from light, for further laboratory analysis.

Determination of semen characteristics

The quality analysis of the fresh semen (and other subsequent analyses) of each male was performed in a 10 μL-aliquot of semen placed on glass slide and observed by light microscopy, at 400 x magnification. The semen was activated by adding water in the ratio 1:4 (semen:water) to evaluate quality and viability to be cryopreserved.

All the ejaculates used were completely suitable to freezing (100% motile sperm). The rate (%) and duration (seconds) of sperm motility were measured. The motility was measured subjectively as the percentage of spermatozoa showing progressive motility by light microscopy at 400 x magnification. The duration was evaluated under the same conditions: a stopwatch was started at the time of addition the activation agent and stopped when 10% of sperm were still moving, and presented as seconds (s) (MILIORINI et al., 2011).

Semen samples (n = 20 males/each treatment) were collected and diluted into two cryoprotectant solutions, in the proportion of 1:4 (semen: Solution dilutive) each of the prepared (stabilized) and composed of follows: Solution A: BTS (5%) + Methanol (7.5%) + 100 mL H_20; Solution B: BTS (5%) + DMSO (7.5%) + 100 mL H_20. The diluted semen was evaluated as for the rate and duration of sperm motility in order to check the toxicity of the cryoprotectant.

An aliquot with approximately 10 µL of the homogenized mixture: fresh semen + solutions (A and B), was placed on a glass slide. Then the motility was activated by adding 4 µL of distilled water. After homogenizing the mixture, we determined the rate (%) and duration (s) of sperm motility. The entire procedure was performed at room temperature (28ºC) simulating field conditions.

Soon after the dilution by cryoprotectant solutions (pre-freezing), the samples were stored in 0.5 mL-Straws, generating a total of four straws/semen sample for each treatment. The straws were transferred to a liquid nitrogen vapor tank (Taylor-Wharton, model CP 300, 'dry shipper') and maintained in vertical position. Twenty-four hours later they were transferred to a nitrogen tank (Cryometal, model DS-18) at -196ºC.

Thawing was carried out after a week of freezing and consisted of removing the straws from the tank, and immersion in water bath at 60ºC for eight seconds. Then, we analyzed the rate and duration of sperm motility according to the method employed for the fresh semen (MILIORINI et al., 2011).

Statistical analyses

The semen from Thailand tilapia was analyzed by sperm motility (%) and duration (s) to evaluate in a factorial arrangement the effect of four oil sources in the diet, and two cryoprotectants, in two moments, before freezing and after thawing. The results were subjected to an analysis of variance, and when significant, the effect of the oil sources and the interaction oil sources x cryoprotectants was checked by Duncan's test at 5% level, using the software SAS (2007).

Results and discussion

The water temperature during the study was 28.23 ± 0.63ºC, and the pH and dissolved oxygen were 7.25 ± 0.58 and 5.23 ± 0.85 mg L^{-1} respectively (NAVARRO et al., 2012).

No significant difference ($p > 0.05$) was found for the final weight, total length, rate and duration of motility of fresh sperm in the different treatments (Table 2).

Table 2. Final weight, length and quality of 'fresh sperm' Thailand tilapia in the different treatments (n = 20).

Different oil sources	Final weight (g)	Length (cm)	Sperm motility (%)	Duration of motility (s)
Fish	281.20 ± 51a	25.93 ± 1.59a	100.00 ± 0a	124.80 ± 0.75a
Corn	297.00 ± 54a	32.57 ± 1.22a	89.00 ± 4a	108.60 ± 0.70a
Linseed	291.25 ± 72a	25.80 ± 1.76a	100.00 ± 0a	123.60 ± 0.66a
Soybean	294.20 ± 40a	28.32 ± 1.52a	93.00 ± 11.54a	154.20 ± 0.74a

Values correspond to mean ± sd; Means in the same column with different superscripts are significantly different according to Duncan test ($p < 0.05$).

There was a significant difference ($p < 0.05$) in the rate of sperm motility of the oil sources, fish, linseed and corn, with the same cryoprotectant DMSO, before freezing (Table 3).

The treatment with linseed and fish oil presented a significantly ($p < 0.05$) longer duration of motility, whereas the treatment with soybean oil led to a shorter duration, before freezing (Table 3). After thawing, fish fed diet supplemented with fish oil presented a significantly ($p < 0.05$) higher rate and duration of sperm motility. The use of the cryoprotectant DMSO before freezing promoted an extended duration of motility regardless of the oil source, but led to a lower rate and duration of motility after thawing when compared with the cryoprotectant methanol (Table 3).

The use of the cryoprotectant DMSO was able to significantly increase ($p < 0.05$) the duration of sperm motility in the treatments with fish, soybean, and linseed oil, unlike the treatment with corn oil, which presented higher values of this parameter when submitted to the cryoprotectant methanol (Table 3).

Table 3. Duration and sperm motility rate of Thailand tilapia fed different sources of oil (FO) and Cryoprotectors (CP) before and after freezing.

		Before Freezing		After Freezing	
Different oil sources (FO)	Crioprotetor (CP)	Motility (%)	Duration (s)	Motility (%)	Duration (s)
Fish	DMSO	90.0 ± 2.00a	218 ± 9a	15.00 ± 5.00bc	20 ± 0.05b
Fish	Methanol	70.0 ± 1.02bc	10 5± 2d	65.00 ± 5.00a	150 ± 0.24a
Soybean	DMSO	70.0 ± 2.40bc	72 ± 6e	8.33 ± 2.88c	16 ± 0.03b
Soybean	Methanol	65.0 ± 1.00c	61 ± 1e	21.66 ± 7.63b	23 ± 0.06b
Linseed	DMSO	80.0 ±1.50ab	218 ± 4a	15.00 ± 5.00bc	19 ± 0.04b
Linseed	Methanol	70.0 ± 1.00bc	182 ± 1b	15.00 ± 3.00bc	19 ± 0.03b
Corn	DMSO	81.0 ± 2.50ab	151 ± 3c	22.00 ± 7.63b	42 ± 0.03b
Corn	Methanol	80.5 ± 2.00ab	180 ± 1b	15.00 ± 5.00bc	31 ± 0.02b
Value of P - FO		0.0201	0.0001	0.0001	0.0001
Value of P - CP		0.0077	0.0001	0.0001	0.0001
Value of P - FO*CP		0.1109	0.0001	0.0001	0.0001

Means in the same column with different superscripts are significantly different according to Duncan test ($p < 0.05$). FO = Different oil sources, CP = Different Cryoprotectant

After thawing, the cryoprotectant methanol was able to extend the duration of sperm motility of fish in the treatment with fish oil, and this parameter did not varied significantly ($p > 0.05$) in the other treatments according to the type of cryoprotectant.

The cryoprotectant methanol promoted a higher rate of sperm motility, after thawing, of individuals subjected to a diet with fish oil (Table 3).

The lack of significant difference in final weight, total length and reproductive parameters in the fresh semen may be result of diets equivalent in caloric value and in proportion of biomolecules (e.g. carbohydrate, protein, lipid) which ensured both energy and energy reserves for growth and weight gain, substrate for mitochondria in the sperm cells to synthetize ATP, used in flagellar movements, regardless of the oil type. However, the type of lipid source led to differences in the frequency and number of some defects in fresh semen.

The fish oil features a greater amount of omega-3 when compared to vegetable oils. This polyunsaturated fatty acid promotes a greater fluidity and strength to cell membranes (NG et al., 2003; NAVARRO et al., 2012). Furthermore, Oliveira et al. (2006) verified that ω-3 long-chain polyunsaturated fatty acids are important components of phospholipids of biological membranes and have been considered as essential for maintaining physicochemical properties of testicular membranes.

The highest percentage of omega-3 in fish oil probably contributed to a physical change in the characteristics of sperm membrane to ensure a greater strength against damage from ice crystal formation, and thus better reproductive parameters after thawing in relation to the other treatments.

In this way, the presence of an oil source with higher concentration of omega-3 probably is essential to reach higher values of motility before freezing, since during this period the treatments with fish oil presented the highest values for sperm motility, subjected to cryoprotectant DMSO. Possibly, this can be result from the least toxic effect of DMSO and improvement in the maintenance of membrane integrity and mitochondrial function by this cryoprotectant, before freezing

Nevertheless, after thawing, the use of cryoprotectant methanol in fish treated with diet containing fish oil showed the highest values of sperm motility and duration. This indicates that the greater concentration of omega-3 of fish oil, and a consequent greater protection of membranes against ice crystals associated with lower toxicity of methanol to tilapia, and its possible improvement in preserving ATP when compared with DMSO can be considered an effective technique to a greater maintenance of reproductive parameters, verified in this study, after thawing. Ogier de Baulny et al. (1997) tested different cryoprotectants (methanol, DMSO, DMA, glycerol, PG) and concluded that methanol promoted a better preservation of ATP of sperm mitochondria of rainbow trout (*Oncorhynchus mykiss*). Therefore, the results of the present study corroborate Rana and McAndrew (1989) who considered the methanol as the most suitable cryoprotectant for tilapia semen. The methanol, according to Harvey (1983) is the intracellular cryoprotectant most permeable to membranes, but has the highest toxicity, except for tilapia sperm (MILIORINI et al., 2011). Godinho et al. (2003) found a rate and duration of motility of 25% and 155 seconds, respectively, for tilapia semen cryopreserved with methanol and activated with distilled water.

Conclusion

The use of oil sources in the diet for Thailand tilapia may contribute to reduce the total number of sperm abnormalities. Besides that, the use of linseed and fish oil for feeding Thailand tilapia and the cryoprotectant DMSO, before freezing, promotes an improvement in the motility rate and duration of

sperm motility. However, for best reproductive parameters after thawing, it is recommended the fish oil associated with cryoprotectant methanol.

Acknowledgements

To PNPD/CNPq for scholarship and for funding the project.

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Received on March 9, 2013.
Accepted on October 16, 2013.