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Criopreservação do sêmen de Jundiá Amazônico (*Leiarius marmoratus*) e refrigeração de sêmen equino

Stela Mari Meneghello Gheller

Pelotas, 2019

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Banca examinadora:

Prof. Dr. Antonio Sergio Varela Junior (Orientador)
Doutor em Aquicultura pela Universidade Federal do Rio Grande

Dr. Charles Nunes Fróes
Doutor em Aquicultura pela Universidade Federal do Rio Grande

Dra. Eliza Rossi Komninou
Doutora em Biotecnologia pela Universidade Federal de Pelotas

Prof. Dr. João Carlos Deschamps
Doutor em Medicina Veterinária por University Of Illinois at Urbana-Champaign, USA

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“O que passou, passou, mas o que passou luzindo, resplandecerá para sempre”
(Johan Goethe)

Resumo

GHELLER, Stela Mari Meneghello. **Criopreservação do sêmen de Jundiá Amazônico (*Leiarius marmoratus*) e refrigeração de sêmen equino**. 2019. 84f. Tese (Doutorado em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2019.

Este estudo avaliou os efeitos da adição de goma xantana no diluente de resfriamento de sêmen equino e de dois protocolos distintos de criopreservação de sêmen de peixes *Leiarius marmoratus*, sobre parâmetros de qualidade espermática analisados *in vitro*. No experimento realizado nas amostras de sêmen da espécie equina, (n=20) armazenadas sob refrigeração (5°C) os grupos experimentais foram: controle (Kenney) e controle adicionado de goma xantana (0,01%, 0,12% e 0,25%). Parâmetros espermáticos, como motilidade, funcionalidade mitocondrial e integridade da membrana, acrossoma e DNA foram avaliados após 0h, 24h, 48h e 72h de armazenamento. Nos experimentos realizados nas amostras de sêmen dos peixes da espécie *L. marmoratus* (n=8) foram utilizados dimetilsulfóxido (DMSO): 5, 10, 15 e 20% e trealose: 50, 100, 150, 200mM. As amostras foram diluídas (1:9 v/v), congeladas em recipiente de vapor de nitrogênio (*dryshipper*), e armazenadas em nitrogênio líquido a -196°C. Após o descongelamento foram analisados parâmetros de motilidade espermática (CASA), tempo de motilidade, integridade de membrana, DNA e funcionalidade mitocôndria. No experimento realizado nas amostras de sêmen da espécie equina a motilidade espermática diminuiu ao longo do período de resfriamento no grupo que recebeu 0,25% de goma xantana em comparação com o grupo controle (60.5 e 44.73% respectivamente). Nos experimentos realizados nas amostras de sêmen dos peixes da espécie *L. marmoratus*, com o uso de protocolo de criopreservação baseado na utilização de diferentes concentrações de DMSO, quanto as motilidades totais e progressivas, linha curva de velocidade (VCL), linha reta de velocidade (VSL) e linearidade, o tratamento contendo 5% de DMSO apresentou resultados inferiores aos tratamentos contendo 15 e 20% de DMSO (P<0,05). O tratamento contendo 10% de DMSO não diferiu dos demais tratamentos (P>0,05). Já no protocolo baseado no uso da trealose, motilidades totais e progressivas superiores foram observadas nos grupos que receberam as maiores concentrações 150mM (22,9 e 17,7%, respectivamente) e 200mM (31,4 e 26,3%, respectivamente) (P <0,05) quando comparadas aos demais grupos, no entanto, não diferindo do tratamento com trealose 100mM (18,6 e 15,3%, respectivamente). Assim, nas condições testadas nesse estudo a adição de goma xantana não é prejudicial à estrutura espermática equina, apesar de reduzir a motilidade total das células. O DMSO não foi eficiente para preservar a qualidade seminal do sêmen dos peixes da espécie *L. marmoratus*. Além disso o tratamento com trealose em altas concentrações demonstraram resultados superiores quando comparados a outros tratamentos em parâmetros de motilidade in vitro para *L. marmoratus*.

Palavras-chave: célula espermática; equinos; Siluriformes; crioprotetores; refrigeração.

Abstract

GHELLER, Stela Mari Meneghello. **Semen cryopreservation of Amazonian catfish (*Leiarius marmoratus*) and stallion semen cooling.** 2019. 84f. Thesis (Doctor degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2019.

This study assessed the effects on sperm quality when Xanthan gum was added to stallion sperm in a cooling protocol and two cryoprotectants added to fish semen (*Leiarius marmoratus*) in two freezing protocols. The stallion semen (n=20) samples were stored cooled (5°C) using different concentrations of xanthan gum added to the standard extender (Kenney): control (no xanthan added), 0.01%, 0.12%, and 0.25%). Sperm parameters, such as motility, mitochondrial function, membrane integrity, acrosome integrity, and DNA integrity, were analyzed after 0, 24, 48, and 72 hours of storage. In the fish species *L. marmoratus* (n=8), semen samples were diluted in dimethyl sulfoxide (DMSO) at 5, 10, 15, and 20% concentrations and in trehalose at 50, 100, 150, and 200 mM concentrations. Samples were diluted (1:9, v/v), frozen in nitrogen vapor vessel (dry shipper), and stored in liquid nitrogen vessel at -196°C. The semen parameters sperm motility (CASA), motility duration, membrane integrity, DNA, and mitochondrial function were assessed post-thawing. Regarding the cooling protocol, the sperm motility was decreased during cooling process in the group 0.25% xanthan gum when compared to the control group (60.5% and 44.73%, respectively). The cryopreservation protocol using trehalose at 50 mM obtained 15.6 and 9.5 % of total and progressive motilities, respectively, being these results inferior to the treatments containing trehalose at 150 mM (22.9 and 17.7%, respectively) and 200 mM (31.4 and 26.3%, respectively) ($P<0.05$). However, it did not differ from treatment with trehalose at 100 mM (18.6 and 15.3 of total and progressive motilities respectively). In the cryopreservation protocol using DMSO, the parameters total and progressive motilities, velocity curved line (VCL), velocity straight line (VSL) and linearity (LIN) were inferior in the treatment at 5% DMSO concentration when compared to treatments containing DMSO at 15 and 20% concentrations ($P<0.05$). Treatment containing DMSO at 10% did not differ from other treatments ($P>0.05$). Therefore, in these experimental conditions the addition of xanthan gum to stallion sperm is not harmful to the sperm cell structure, despite of reducing total motility. DMSO was not efficient to preserve semen quality and the addition of trehalose at high concentrations exhibited superior results when compared to other treatments in in vitro motility parameters in *L. marmoratus*.

Keywords: cooling; cryoprotectans; Siluriformes; sperm cells; stallion

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Lista de Abreviaturas e Siglas

ANOVA	Analysis of Variance
BTS	Beltsville Thawing Solution
CASA	Computer Assisted Sperm Analysis
CFDA	Carboxifluoresceína
DMF	Dimetilformamida
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
EDTA	Ácido etilenodiamino tetra-acético
FICT	Lectin Conjugate
FURG	Universidade Federal do Rio Grande
H	Horas
IA	Inseminação Artificial
ICB	Instituto de Ciências Biológicas
IP (PI)	Iodeto de Propídio (*Propidium Iodide*)
Kg	Quilograma
Km	Quilômetros
LIN	Linearity
µL	Microlitro
µM	Micromolar
M	Molar
mL	Mililitros
mg/mL	Miligrama por mililitro
MIN	Minuto
mM	Milimolar
n	Número
nm	Nanômetros
NaCl	Cloreto de Sódio

$NaHCO_3$	Bicarbonato de Sódio
P	Valor de *P*
PBS	Buffer salina fosfato
pH	Potencial Hidrogeniônico
PMO	Progressive motility
ReproPel	Núcleo de Ensino e Pesquisa em Reprodução Animal
Rh 123	Rodhamine
ROS	Reactive Oxygen Species
RS	Rio Grande do Sul
S	South
SEM	Standard Error Means
SisBi/UFPel	Sistema de Bibliotecas da Universidade Federal de Pelotas
SP	São Paulo
TMO	Total motility
UFPel	Universidade Federal de Pelotas
USA	United States of America
VAP	Velocity average path
VCL	Velocity curved line
VSL	Velocity straight line
v/v	Volume por Volume
W	West

Lista de Símbolos

~ Aproximadamente

\+ E/Mais

°C Grau Celsius

° Graus

\> Maior

± Mais ou menos

® Marca Registrada

< Menor

’ Minutos

% Porcento

” Segundos

™ Trade Mark

× Vezes

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1 Introdução

As biotecnologias aplicadas à reprodução animal vêm contribuindo para o melhor aproveitamento do potencial reprodutivo e genético nas diferentes espécies de interesse comercial. Biotecnologias relacionadas à conservação do sêmen, tais como a refrigeração e a criopreservação, quando colocadas dentro de um cenário produtivo, podem contribuir com amplos benefícios, principalmente aqueles comumente associados ao uso da inseminação artificial.

Alguns exemplos incluem: redução de custos com a aquisição de animais geneticamente superiores, racionalização do uso do macho reprodutor através da sua utilização para um grande número de fêmeas e, ainda no caso da criopreservação, armazenamento do sêmen por tempo indeterminado, uso na reprodução de animais que perderam a capacidade reprodutiva ou vieram a óbito, controle de doenças venéreas e facilidade no transporte a longas distâncias (PIMENTEL e CARNEIRO, 2008).

Quando refrigerado ou criopreservado, o sêmen é submetido a temperaturas inferiores à fisiológica e com isso ocorre uma redução do metabolismo celular, permitindo sua viabilidade por períodos superiores a 24h no sêmen resfriado, e por tempo indeterminados no sêmen criopreservado (MELO et al., 2005). Entretanto, algumas questões como: a influência do tempo de refrigeração sobre a taxa de prenhez, meios diluentes ideais, acúmulo de radicais livres pelas células durante os processos, recipientes adequados para o envase do sêmen, temperaturas ideais de refrigeração e congelamento e danos ocasionados por choque térmico, por exemplo, precisam ser melhor exploradas, considerando-se particularidades espécie-específicas.

Em sêmen de equinos existe uma grande quantidade de diluentes utilizados no resfriamento, com temperaturas variando entre 5 e 15°C. E esses diluentes estão classificados conforme a capacidade de manutenção da viabilidade espermática em função do tempo. A base dos diluentes utilizados na espécie tem em sua constituição a água, tampões, açúcares e substâncias não iônicas, podendo ainda ser acrescido

de antibióticos (CANISSO et.al. 2008), com manutenção da qualidade seminal e taxas de fertilidade semelhantes ao sêmen fresco por até 24h de refrigeração.

No processo de conservação de sêmen, muito ainda precisa ser estudado quando consideramos a eficiência de soluções de resfriamento e crioprotetores. Neste sentido, visando aumentar a eficiência dos processos de conservação seminal, estratégias como a pesquisa de novos meios diluentes, curvas de temperatura de resfriamento, além da adição de protetores de membrana às soluções utilizadas nos processos podem ser consideradas (WATSON, 2000). Além disso, o volume dessas soluções para uma concentração final adequada, bem como sua toxicidade para as células são pontos importantes, uma vez que variam entre as espécies e até mesmo entre indivíduos (VIVEIROS et al., 2014).

Estudos *in vitro* utilizados atualmente permitem avaliar os efeitos de diluentes de resfriamento e de soluções crioprotetoras sobre a integridade e a funcionalidade das diferentes estruturas que compõem a célula espermática, aumentando a confiabilidade do uso dessas soluções quando utilizadas em escala comercial.

Quando comparados a estudos realizados em espécies de mamíferos, se observa que em nível de piscicultura, os estudos relacionados à conservação do sêmen são mais recentes, com várias questões sobre a sua utilização ainda por serem respondidas. Isto, em parte, deve-se à grande variedade de espécies de peixes que são utilizadas comercialmente, com isso havendo muitas vezes a necessidade de se buscar protocolos específicos para grupos específicos de peixes.

Em vista do já descrito, na espécie equina e de peixes *Leiarius marmoratus* o presente estudo teve como objetivos os descritos abaixo:

Objetivo Geral

Avaliar os efeitos do aumento da viscosidade provocado pela utilização de goma xantana adicionada ao diluente Lake de sêmen equino sobre parâmetros de qualidade espermática, ao final de um período de 72h sob refrigeração a 5°C e desenvolver um protocolo de criopreservação de sêmen de peixes *Leiarius marmoratus* utilizando duas abordagens: uma utilizando dimetilsulfóxido como crioprotetor interno e outra utilizando trealose como crioprotetor externo de membrana.

Objetivos Específicos

1) Desenvolver um protocolo de criopreservação seminal para a espécie *Leiarius marmoratus*, devido sua importância na piscicultura nacional.
2) Testar meios diluentes com consistência mais espessa para refrigeração de sêmen equino, durante o período e temperatura de refrigeração proposto para a espécie.
3) Avaliar *in vitro* a estrutura morfológica, funcional e cinética espermática das células submetidas a esses dois métodos de processamento para conservação de sêmen.

2 Revisão da Literatura

2.1 Aquicultura

Define-se por aquicultura o processo de produção em cativeiro, em condições controladas, de organismos que vivem em ambientes predominantemente aquáticos. O Brasil é um país privilegiado devido a quantidade de água doce disponível e a variabilidade de espécies com potencial produtivo/comercial (BALDISSEROTTO e GOMES, 2005).

Entretanto essas espécies se encontram em declínio populacional, devido a sobrepesca, urbanização, mudanças fluviais e construção de barragens hidrelétricas, que interferem no comportamento migratório, provocando a formação de populações com alto grau de parentesco e restrição de fluxo genético, fatores que podem ocasionar uma diminuição da heterogeneidade genética, podendo levar a ameaça de extinção (CAROLSFELD et al., 2003).

Além destes fatores, a poluição por efluentes industriais, esgotos domésticos e rejeitos da mineração mudam as características naturais das águas como a salinidade, concentração de íons e pH, afetando diretamente a ativação e motilidade do esperma dos peixes e consequentemente comprometendo sua reprodução (BONISŁAWSKA et al., 2015; DZIEWULSKA e DOMAGAŁA, 2013).

Entre as espécies nativas estão os Siluriformes (CAMPOS, 1998) e dentro desta classificação destaca-se o jundiá amazônico (*Leiarius marmoratus*), de ocorrência ampla nas bacias dos rios Amazonas e Orinoco e que apresenta migração reprodutiva durante a época de chuvas (Figura 1). O jundiá amazônico é um peixe de couro, possui grande importância comercial, sendo uma das promessas da piscicultura brasileira. Sua reprodução quando realizada em cativeiro necessita de indução hormonal para a produção de gametas, pois na ausência do estímulo da migração, não conseguem completar a gametogênese.

Aliado a isto, se tem a comercialização de híbridos entre animais desta família. A produção, manejo e comercialização desses produtos é realizada geralmente sem

controle e monitoramento, o que prejudica não só a indústria piscícola como também representa um risco biológico para as populações naturais, uma vez que pode haver introgressão genética e perda de variabilidade das espécies parentais nas populações naturais (HASHIMOTO, 2011).

Neste contexto, se faz necessário estudos relacionados a reprodução e desenvolvimento dessa espécie nativa, seja para fins comerciais ou manutenção da variabilidade genética (SALARO et al., 2003). As técnicas de criopreservação espermática podem contribuir nesse cenário, atendendo a necessidade de manutenção de banco de germoplasma para espécies que possam vir a ser ameaçadas de extinção, e também contribuindo para a redução do número de reprodutores em pisciculturas, reduzindo gastos com alimentação e consequentemente da produção.

Espécies em Estudo: Jundiá Amazônico: *Leiarius marmoratus*

Taxonomia do Jundiá Amazônico (*Leiarius marmoratus*)

Reino:Animalia

Filo:Chordata

Classe:Actinopterygii

Subclasse:Neopterygii

Infraclasse:Teleostei

Superordem:Ostariophysi

Ordem:Siluriformes

Família: Pimeloidae

Gênero: *Leiarius*

Espécie: *L. marmoratus*

Figura 1 – Peixe Jundiá Amazônico (*Leiarius marmoratus*)

2.1.1 Criopreservação de Sêmen de Peixes

A criopreservação de sêmen de peixes é uma técnica de grande interesse para a piscicultura comercial, podendo colaborar para a difusão de material genético, otimização do melhoramento genético, redução do número de machos no plantel, seleção de reprodutores, minimização dos efeitos de assincronia reprodutivas entre machos e fêmeas e melhor aproveitamento seminal, (SUQUET et al., 2000). Possibilita também o atendimento através de banco de germoplasma, na conservação da variabilidade genética dos animais da natureza e troca de material genético entre pisciculturas comerciais produtoras de alevinos (MARTÍNEZ-PÁRAMO et al., 2009; CABRITA et al., 2010).

O sucesso da biotecnologia de criopreservação de sêmen, é dependente da composição dos meios diluentes, concentração, tipo/classe química dos crioprotetores e método de congelamento (SANSONE et al., 2002; DEGRAAF e BERLINSKY, 2004). O desenvolvimento de uma solução crioprotetora para uma espécie de peixe necessita de ensaios para a avaliação de seus eventuais efeitos tóxicos e sua influência sobre a integridade e funcionalidade da célula espermática. Tanto a escolha do diluente/crioprotetor, quanto suas proporções devem ser determinadas para cada espécie de peixe (VIVEIROS et al., 2014).

A solução diluidora deve ser adicionada, para favorecer a manutenção da célula espermática durante a curva de resfriamento ante e após a criopreservação (GARCIA et al., 2016), fornecendo suporte metabólico, controle de pH da osmolaridade do meio (LAHNSTEINER e RADNER, 2010). Um exemplo de meio diluente amplamente utilizado em peixes de água doce é o Belstville Thawing Solution – BTS (PURSEL e JOHNSON, 1975), inicialmente desenvolvido para refrigeração e conservação de sêmen de suínos, e que tem demonstrado bons resultados quando utilizado sozinho

para a refrigeração ou associado a crioprotetores na criopreservação. Isto se deve à sua composição química e osmolaridade, que não promovem a ativação seminal precoce em amostras de sêmen de peixes. O BTS como meio diluente associado a diferentes crioprotetores com resultados satisfatórios tem sido reportado em diversas espécies de peixes como: *Brycon insignis* (VIVEIROS et al., 2012); *Brycon nattereri* (OLIVEIRA et al., 2007; VIVEIROS et al., 2012), *Brycon orbignyanus* (VIVEIROS et al., 2015); *Colossoma macropomum* (VARELA JUNIOR et al., 2012; GARCIA et al., 2015; VARELA JUNIOR et al., 2015); *Leporinus obtusidens* (VIVEIROS et al., 2008) e *Piaractus mesopotamicus* (STREIT JR et al., 2006).

Os crioprotetores tem como função promover a desidratação celular e com isso evitar a formação de cristais de gelo intracelular, prevenindo assim o rompimento das membranas celulares. Cada crioprotetor possui uma formulação química sendo feita a escolha a partir de sua baixa toxicidade e alta solubilidade em água, além da capacidade de penetrar a membrana espermática facilmente, e se ligar com moléculas de água (CHAO E LIAO, 2001), sendo classificados em intracelulares (permeáveis) e extracelulares (impermeáveis).

Os crioprotetores intracelulares são solventes orgânicos de baixo peso molecular (RALL et al., 1984). Agem substituindo parcialmente e ligando-se ao hidrogênio das moléculas de água no interior da célula (JAIN e PAULSON, 2006), aumentando assim a viscosidade da solução de congelamento, com isso reduzindo o ponto de congelamento e diminuindo a ocorrência de danos tóxicos e osmóticos (KASAI, 1996).

A eficiência desses crioprotetores varia em função da estrutura a ser criopreservada, além da classe, concentração e tempo de exposição utilizados para a estabilização antes de ser submetido a criopreservação (FULLER et al., 2004). Os mais citados para protocolos de criopreservação em peixes de água doce são: etilenoglicol, glicerol, metanol, propilenoglicol, dimetilsulfóxido (DMSO), dimetilacetamida (DMA) (HORVATH e URBANYI, 2000; VIVEIROS et al., 2014; VIVEIROS et al., 2015; VARELA JR et al., 2012), com destaque para peixes de água doce nativos, o Dimetilsulfóxido (DMSO) em concentrações de 5 a 15%.

Os crioprotetores extracelulares são nada mais do que diversos tipos de açúcares e têm sido incluídos aos diluentes para criopreservação de sêmen por atuarem como substratos de energia, componentes osmóticos e agentes crioprotetores, em função de seu alto peso molecular, contribuindo para o equilíbrio

osmótico (HOLT, 2000). Crioprotetores não penetrantes como os açúcares (dissacarídeos), protegerem a membrana celular a partir do volume celular no congelamento e descongelamento (SILVA et al., 2015), reduzindo o volume de água passível de ser congelada no interior da célula (AISEN et al., 2002).

O uso de açúcares em diversas espécies permitiu concluir que os dissacarídeos, como sacarose (glicose + frutose) e trealose (glicose + glicose), são mais efetivos na função de estabilizar a bicamada lipídica da membrana espermática do que os monossacarídeos (DE LEEUW et al., 1993) e conferem maior flexibilidade à membrana espermática, tornando-a mais resistente aos danos causados pelo choque térmico (AISEN et al., 2005; BERLINGUER et al., 2007). Estes dissacarídeos auxiliam na redução da excessiva desidratação celular, reduzem a formação de cristais de gelo e, por consequência, a diminuição das crioinjúrias nas organelas celulares. Além disso, capacidade antioxidante tem sido atribuída a esses açúcares, conferindo maior proteção à membrana celular contra a ação de radicais livres (BUCAK et al., 2007).

Muitas vezes são utilizadas associações de crioprotetores internos e externos com o intuito de se reduzir a toxicidade e os danos osmóticos, entretanto, estudos realizados demonstram que apenas o uso de trealose é capaz de manter taxas de qualidade seminal pós descongelamento, sem adição de crioprotetor interno. Em suínos, esses resultados foram alcançados com concentração final de trealose 100mM (ATHURUPANA et al., 2015). Em peixes, o mesmo foi descrito em sêmen de tambaqui (150mM) e em *Pagrus major* (100mM) (LIU et al., 2015).

A curva de congelamento a ser utilizada durante o processo de criopreservação deve ser de fácil aplicação comercial. Um método que vem apresentando boa aceitação pelos resultados e praticidade é a curva de congelamento realizada com o auxílio do botijão de nitrogênio dry-shipper (TAITSON et al., 2008). Além de ser considerado por outros autores como um método de congelamento prático e seguro (VARELA JR et al., 2015), esse equipamento possui variação muito pequena de temperatura, e quando realizado seu uso de forma adequada (considerando local e volume de nitrogênio), a taxa de congelamento atinge queda de temperatura de 27,5 – 40 °C/min, até sua estabilização -60°C (TAITSON et al., 2008; CARNEIRO et al., 2012; MARIA et al., 2015). Devendo ser transferidos para um botijão de nitrogênio líquido com temperatura de -196°C após 24h permanecendo até o momento do descongelamento.

Mesmo diante do sucesso de diferentes protocolos de criopreservação para sêmen de peixes de água doce nativos, os mesmos ainda não haviam sido testados em *L. marmoratus*, bem como não se tinha o conhecimento de qual a concentração de crioprotetor, diluente e curva de congelamento ideais para esta espécie. Até o nosso conhecimento este é o trabalho inicial na criopreservação seminal de jundiá amazônico, espécie de grande importância comercial na piscicultura brasileira atual. Análises *in vitro* realizadas após o descongelamento das amostras de sêmen criopreservadas, considerando parâmetros de estrutura, de qualidade e de funcionalidade das células espermáticas e auxiliaram no desenvolvimento de um protocolo eficiente de criopreservação seminal para a espécie. Tais análise incluíram: integridade de membrana, de DNA, funcionalidade de mitocôndria, taxa de motilidade espermática e tempo de motilidade.

2.2 Refrigeração Seminal

As biotecnologias aplicadas a reprodução animal, contribuem para o progresso da reprodução em diversas espécies, mas tem grande destaque quando se trata de animais de produção. Muitas pesquisas já foram e continuam sendo realizadas com a finalidade de desenvolver, aprimorar e difundir essas técnicas reprodutivas. Dessas técnicas a IA está entre as mais utilizadas pela viabilidade econômica e facilidade de implantação, além de ser a base para a realização de outras técnicas como a transferência de embriões.

A técnica de inseminação artificial, pode ser realizada através do uso de sêmen fresco, criopreservado, ou resfriado, sendo que as duas últimas utilizam a curva de refrigeração para redução gradativa de temperatura. Essa redução de temperatura é necessária para se conseguir manter o potencial fertilizante por períodos mais longos, devido a redução do metabolismo das células espermáticas (BATELLIER et al., 2001).

No processo de refrigeração seminal se faz necessário o uso de diluentes de resfriamento que devem assegurar em sua composição uma fonte de energia (açucares), tampões (manutenção do pH), pressão osmótica adequada, antibióticos (evitar o crescimento microbiano) e crioprotetores externos (ex: gema de ovo e leite), afim de reduzir a ocorrência de choque térmico. Esses meios diluentes são adicionados ao sêmen fresco para garantir a manutenção das células e prolongar sua sobrevivência durante a refrigeração e o transporte, além de aumentar o volume da

dose inseminante, facilitando a análise e o percurso dos espermatozoides no trato reprodutivo da fêmea (KARABINUS et al., 1997).

No processo de resfriamento, a célula espermática está vulnerável ao choque térmico entre 15 e 5°C, faixa em que um ritmo muito rápido de refrigeração induz a danos irreversíveis a sua motilidade, integridade estrutural e capacidade fertilizante (WATSON, 2000). Estes danos podem ser minimizados através do controle da taxa de refrigeração do sêmen ou pela adição de componentes protetores ao diluente. Isso ocorre, pois, envolvendo toda a célula espermática, existe a membrana plasmática que é composta por uma dupla camada de lipídios, contendo em sua estrutura proteínas e carboidratos. As membranas espermáticas são as estruturas mais afetadas pelo choque térmico (AMANN e GRAHAM, 1993).

Mesmo com o uso de meios de refrigeração a capacidade fertilizante dos gametas pode reduzir de forma constante, a uma taxa de 3-6% por dia, após 3 dias de refrigeração (YOSHIDA, 2000). Isso pode ser explicado pois mesmo com a desaceleração das reações enzimáticas, o metabolismo celular ainda continua ocorrendo, ocasionando danos pela ação de espécies reativas de oxigênio. (CHATTERJEE e GAGNON, 2001; FUNAHASHI e SANO, 2005; MARTÍN-HIDALGO et al., 2011; ORZOŁEK et al., 2013). Esta não tem sido relacionada ao aumento de sua produção durante a refrigeração, mas sim com as reações enzimáticas que as controlam que estão mais lentas. Logo, mesmo com uma produção baixa, em temperaturas reduzidas podem ocorrer danos espermáticos devido à carência enzimas antioxidantes (HU et al., 2009).

Segundo HOLT (2000), deve-se considerar na refrigeração a distribuição das células no meio diluente, sendo essa fundamental para a manutenção da funcionalidade e viabilidade espermática, não devendo se aglutinarem e/ou sedimentarem. Para isso se faz necessário durante o período de refrigeração, a adequada homogeneização das amostras quando utilizados diluentes com baixa viscosidade (líquidos), que representam hoje quase que a totalidade dos diluentes comerciais disponíveis.

Estudos na tentativa de aumentar a viscosidade do meio diluente foram desenvolvidos, ficando esse meio com um aspecto mais sólido em temperatura de refrigeração entre 5 e 15°C, e voltando ao estado liquido em temperatura próxima à corporal (aproximadamente 37°C, para mamíferos). Segundo LÓPEZ-GATIUS et al. (2005), esse meio mais viscoso favorece a disposição das células no meio e além

disso apresenta como vantagem não haver a necessidade de homogeneização, devido ao meio não permitir a sedimentação celular. Outras vantagens incluem o favorecimento da ação dos tampões, e também a atuação como protetor externo durante o resfriamento.

A goma xantana produz um meio viscoso e tem como característica ser um biopolímero natural de alto peso molecular produzida pelo microorganismo *Xanthomonas campestres* (GHARIBZAHEDI et al., 2012) que aumenta a viscosidade na presença de sais, esse aumento da viscosidade é devido a interações moleculares que aumentam o tamanho efetivo da macromolécula e consequentemente seu peso molecular (GARCIA-OCHOA et al., 2000), tendo boa estabilidade durante o armazenamento em uma ampla faixa de temperatura de (4 e 93ºC) e pH (XIONG et al., 2013).

A goma xantana, adicionada a criopreservação de células espermáticas de ovinos (GASTAL et al., 2017), demonstrou possuir capacidade antioxidante. Essa age removendo os produtos produzidos pelo metabolismo (radicais livres) e inibindo a peroxidação lipídica (XIONG et al., 2013). A atividade antioxidante deste oligossacarídeo pode ser devida a presença de resíduos de ácido piruvato e a sua capacidade de reduzir o teor de açúcar (XIONG et al., 2013). Devido a goma xantana apresentar essas propriedades se desenvolveu a hipótese de que a sua adição em meios diluentes para refrigeração a 5°C de sêmen equino, pode ser favorável à manutenção das células espermáticas.

2.2.1 Refrigeração de sêmen equino

Os equinos têm grande importância na prática de esportes, na lida no campo, na saúde humana (equoterapia), além de estar envolvidos em atividades de lazer, equitação, cavalgadas, entre outros (SILVA et al., 2011). As biotecnologias aplicadas a reprodução equina, atuam como importante ferramenta a serviço da indústria equina mundial, atuando como recurso para o melhoramento genético.

A inseminação artificial é realizada predominantemente com o uso de sêmen resfriado (LOMMIS, 2006), devido amostras de sêmen refrigerado apresentarem resultados semelhantes de fertilidade com o sêmen fresco quando utilizados por um período de 24h após a coleta (MELO et al., 2005). Quanto ao uso de sêmen criopreservado, a variação ainda é muito grande entre os animais nos protocolos de

criopreservação, e a proporção de garanhões que não possuem resposta satisfatória é em torno de 20 a 40% (VIDAMENT et al., 1997), mesmo com os avanços recentes da pesquisa.

O sêmen resfriado apresenta algumas vantagens quando utilizado, como a redução com os gastos inerentes ao transporte e hospedagem de animais, a diminuição do estresse pelo transporte, redução dos riscos com acidentes e aquisição de doenças resultantes da exposição a patógenos de um novo ambiente (BRINSKO e VARNER, 1992).

Segundo BATELIER et al. (2001), o sucesso do uso de sêmen refrigerado na inseminação artificial depende de fatores como: taxa de resfriamento, temperatura de armazenamento, dose inseminante, número de inseminações, manuseio do sêmen, composição do diluente entre outros. Melhores resultados nestes protocolos de manutenção seminal podem favorecer a indústria equina, pois permitem que seja realizado apenas o transporte de amostras e não do reprodutor entre centrais de reprodução.

Na tentativa de melhorar a conservação do sêmen refrigerado nesta espécie muitos diluentes e protocolos com diferentes curvas de resfriamento e temperaturas foram testados, além da adição a esses meios diluentes de componentes específicos com o intuito de preservar as diferentes estruturas espermáticas como a integridade de membrana, bem como de evitar o estresse oxidativo, e de preservar a motilidade espermática.

Os crioprotetores extracelulares já testados e mais utilizados são a gema de ovo e o leite, pela sua praticidade e eficiência na proteção durante o armazenamento entre 4ºC - 8ºC (BATELLIER et al., 2001). O leite integral, ou desnatado também tem sido utilizado por muito tempo como meio diluidor de sêmen equino (KENNEY et al., 1975). Segundo SALAMON e MAXWELL (2000), o sucesso deste diluente tem relação com a fração proteica, que atua como um tampão contra variações do pH, e como agente quelante. Além disso apresenta proteção parcial contra danos ocasionados na curva de resfriamento. Segundo, BATELLIER et al. (2001) relataram que a proteção conferida pelos componentes do leite estaria relacionada aos seus efeitos antioxidantes. E a gema de ovo confere proteção contra o choque térmico aos espermatozoides. Essa ação protetora deve-se às lipoproteínas de baixa densidade (LDL) (AMANN e GRAHAM, 1993), que permanecem firmemente ligadas aos espermatozoides e auxiliam na estabilização da membrana.

Em sêmen de equinos existe uma grande quantidade de diluentes utilizados no resfriamento, com temperaturas de 5 e 15°C. E esses diluentes estão classificados conforme a capacidade de manutenção da viabilidade espermática em função do tempo. A base dos diluentes utilizados na espécie tem em sua constituição a água, tampões, açucares e substancias não iônicas, podendo ainda ser acrescido de antibióticos. Alguns autores dividem os diluidores em quatro grupos: salinos, com gema de ovo, com leite e derivados e os que apresentam albumina sérica bovina (CANISSO et al., 2008). Se tem também a opção de diluidores comercializados no Brasil como: EZ – Mixin® (CST), Max Sêmen® (Agrofarma); Botu-Sêmen® e Botu-turbo® (Biotech Botucatu) Equimix® (Nutricell), sendo que todos utilizam leite e ou derivados em sua composição (RAPHAEL, 2007).

A adição do polímero de goma xantana, até a realização de nosso estudo ainda não havia sido testada como aditivo no diluente de resfriamento para essa espécie na temperatura de 5°C por 72h.

3 Artigos

3.1 Artigo 1

The effects of xanthan gum on equine sperm quality during cooling storage
[*Efeitos de xantam gum em qualidade de esperma equino durante armazenamento resfriado]*

Stela Mari Meneghello Gheller, Carine Dahl Corcini, Fernanda Carlini do Santos, Geórgia Cruz Tavares, Vitória Costa, Bruna Curcio, Carlos Eduardo Nogueira, Antonio Sergio Varela Junior

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The effects of xanthan gum on equine sperm quality during cooling storage

[*Efeitos de xantam gum em qualidade de esperma equino durante armazenamento resfriado*]

S.M.M. *Gheller*[1], C.D. *Corcini*[2*], F.C.C. *Santos*[1,2], G.C. *Tavares*[2], V.G.G. *Costa*[2], B.R. *Curcio*[2], C.E.W. *Nogueira*[2], A.S. *Varela Junior*[2,3]

[1]Aluno de pós-graduação - Universidade Federal de Pelotas - Pelotas, RS

[2]Universidade Federal de Pelotas - Pelotas, RS

[3]Universidade Federal de Rio Grande - Rio Grande, RS

ABSTRACT

This study was designed to evaluate the possible benefits of adding xanthan gum to a standard extender for equine through *in vitro* analyzes of sperm quality. Semen was collected four times from five different stallions (n= 20 samples) and subjected to cooled storage under different conditions: control (only standard extender) and three different concentrations of xanthan gum (0.01%, 0.12%, and 0.25%) supplemented to the extenders. Sperm parameters, such as motility, mitochondrial functionality, and membrane, acrosome, and DNA integrity were measured after 0h, 24h, 48h, and 72h of sperm storage at 5ºC. Our observations indicated that sperm motility declined with longer cooling period with the 0.25% xanthan gum supplementation group compared with the control group. Other parameters, such as mitochondrial functionality and membrane and acrosome integrity also declined for all treatments during storage; however, no differences were observed between xanthan gum and control groups. DNA integrity did not significantly change during the storage. In conclusion, the addition of xanthan gum to equine semen extender is not harmful to the sperm structure, despite reducing the sperm motility.

Keywords: cooled, stallion, semen, spermatozoa, storage

RESUMO

Esse estudo foi desenvolvido para avaliar os possíveis benefícios de acrescentar xanthan gum a um extensor padrão através de analises in vitro de qualidade de esperma. Semen foi coletado quatro vezes de cinco garanhões diferentes (n = 20 amostras) e submetido a armazenamen to resfriado em diferentes condições: controle (apenas extensor padrão) e três diferentes concentrações de xanthan gum (0,01%, 0,12% e 0,25%) suplementado aos extensores. Parâmetros dos espermatozoides, como mobilidade, funcionamento mitocondrial e integridade de membranas, acrossomos e DNA forma medidos após 0h, 24h, 48h e 72h de armazenamento a 5oC. Nossas observações indicaram que motilidade reduziu com armazenamento resfriado prolongado no grupo de 0,25% de suplementação de xanthan gum comparado ao grupo controle. Outros parâmetros, como funcionalidade mitocondrial e integridade de membrana e acrossomos também reduziu em todos os tratamentos durante o armazenamento, no entanto não foram detectadas diferenças significativas entre grupos tratados e grupo controle. Integridade de DNA não mudou

significativamente durante armazenamento. Em conclusão, a adição de xanthan gum a extensor de sêmen equino não é danosa à estrutura do espermatozoide apesar de reduzir motilidade.

Palavras-chave: resfriamento, garanhão, sêmen, espermatozoide, armazenamento

INTRODUCTION

Artificial insemination in horses is widely practiced throughout the world by processing equine semen for cooled transport. In the cooled transport conditions, inseminations can be carried out for the equine semen for up to 48h when stored at 5ºC (Scott Weese *et al.*2011). However, this reduction in temperature causes structural changes in the spermatozoa, which can compromise their functional integrity, either by thermal shock or by the production of free radicals from spermatic catabolism (Watson, 2000). The thicker diluents maintain the homogeneity of the sperm cells in the diluent medium, and metabolically less active, being able to resurrect in a smaller formation of ROS (Lopez-Gatius *et al.*, 2005; Nagy *et al.*, 2002). Xanthan gum has already been demonstrated in other studies with sperm cells (Gastal *et al.*, 2017) that may exert antioxidant effect. And second, due to the antioxidant activity of xanthan oligosaccharides may be due to the presence of pyruvate acid residues and to their capacity of reducing sugar content (Xiong *et al.*, 2013).

Extenders have been used for equine semen refrigeration, and they usually contain milk. However, there is a considerable variation in fertilization rates using refrigerated semen samples transported in milk-based extenders (Scott *et al.*, 2011). Studies to improve the conservation of refrigerated semen have focused on modifying extenders using a combination of various components, such as sugars, electrolytes, buffers, egg yolks, milk, antioxidants, and adjuvants (Scott *et al.*, 2011). The addition of such components was meant to help maintain membrane integrity, prevent oxidative stress, and preserve sperm motility (Samper *et al.*, 2002).

In addition to the above-mentioned factors, sperm cell survival and functional integrity are also dependent on the spatial arrangement of the refrigerated samples. This is achieved through homogenization that helps avoid cell sedimentation (Holt, 2000). The beneficial effects of addition of gelatin to tris / yolk-based cooling diluents and milk powder on refrigeration demonstrated beneficial effects in studies with rabbits (Nagy *et al.*, 2002; Lopez- Gatius *et al.*, 2005), sheep (Yaniz *et al.*, 2005), and goats (Salvador *et al.*, 2006).

Xanthan gum, a naturally occurring, high molecular weight biopolymer produced by *Xanthomonas campestris*, increases the viscosity (in the presence of salts) and stability during storage over a wide temperature range (4 and 93ºC). In addition, xanthan gum has the potential to reduce cellular damage during the cooling process by acting as an external cryoprotectant by increasing the viscosity of the extender medium and by having antioxidant effects. There is no study on the effect of diluents supplemented with xanthan gum to enhance the storage properties of stallion semen. Thus, the objective of this work was to evaluate the effects of adding xanthan

gum to the equine cooling extender, and how that affects sperm parameters *in vitro* during storage at 5ºC for 72h.

MATERIALS AND METHODS

We performed four semen collections from five different fertile stallions (n= 20 samples) belonging to Crioulo and Quarter horse, age 4−6 years, during the reproductive season. Semen ejaculates were collected utilizing a teaser mare and artificial vagina (Hannover, Minitüb™), and processed at the Núcleo de Ensino e Pesquisa em Reprodução Animal - REPROPEL of Federal University of Pelotas - UFPel. The experiments were carried out after obtaining due approval from the "Committee of Ethics in Animal Experimentation," Federal University of Pelotas (UFPel), S.No. 1946.

The base diluent used was Kenney extender (1975), having pH 6.9, and osmolality of 380mOsm/Kg. The diluent was prepared the day before semen collection, and stored at –5°C. It was reheated in a water bath to achieve the temperature of 33°C immediately before use. The semen samples were diluted in the base extender (1:1, v/v) at the time of collection and immediately evaluated for motility and sperm vigor. The ejaculates had sperm motility ≥70% and sperm disease < 30%. The final dilution was performed after determining the sperm concentration of the samples diluted in formaldehyde (Bearden e Fuquay, 1997) B). The final dilution used was 50×10^6 viable spermatozoa/mL in the following treatments at room temperature: Kenney only (control group) and Kenney containing 0.01%, 0.12%, and 0.25% xanthan gum (treatment groups). The cooling curve was performed using a conditioning box (Koolmate, Minitube, Ge) with a cooling rate of 0.3–0.5°C/min until a temperature of 5°C was achieved followed by storage for a period of 72h.

Prior to the analysis, all conditioned samples that were cooled for 0h, 24h, 48h and 72h, were heated in a water bath (5min/37°C). Subsequently, 200 cells were counted per sample, except in the case of sperm motility experiments. For sperm motility, 10µL of sperm, maintained at 37°C on a heated plate, was examined using phase-contrast optical microscopy at 200x magnification (Bearden e Fuquay, 1997).

The functionality was evaluated as described by (Garner *et al.*, 1997). Briefly, 20µL of the semen was added to 13mM Rhodamine 123 (Rh123, R8004), followed by Propidium Iodide (PI, P4170). The samples were incubated for 5min in the dark in 24ºC and immediately examined microscopically. The cells with intense green fluorescence in the intermediate part were considered functionally active, whereas the cells with less intense or without any green

fluorescence in the intermediate part were considered functionally inactive.

The membrane integrity was measured according to the protocol described by (Harrison and Vickers, 1990). Briefly, 20µL of semen was added to carboxyfluorescein diacetate (CFDA, C4916), followed by Propidium Iodide (PI, P4170) in formaldehyde and sodium citrate, and incubated for 5min in the dark in 24ºC. Cells showing green fluorescence were considered intact, while cells with red or green/red fluorescence were considered damaged.

The acrosome integrity (Kawamoto *et al*., 1999) was evaluated on semen smears prepared using 20µL of semen. Propidium iodide (PI, P4170) (20µL) was added to this smear, the slides were dried, immersed in absolute ethyl alcohol (459844–1L) for 5min, washed in PBS, followed by the addition of 20µL of *Arachis hypogaea*- FITC Lectin Conjugate (20mg/mL). The slides were subsequently washed in deionized water and drained in a dark room in 24ºC. The cells with intact acrosome emitted green fluorescence, whereas those with damaged acrosome appeared rough with vacuoles, and did not emit green fluorescence.

The DNA integrity was evaluated using the Acridine Orange dye (A6014), as per the protocol of (Evenson *et al*., 1999). Briefly, 10µL of TNE (0.01M Tris-HCl, 0.15M NaCl, 0.001M, EDTA, pH 7.2) was added to 20µL of semen. After 30s, 100µL of Triton (1x) was added, and after another 30s, 50µL of Acridine Orange (2mg/mL in deionized water) was added, followed by incubation for 5min in 24ºC. The cells with normal (double-stranded) DNA had green fluorescence, whereas those with red fluorescence were considered to have denatured (single-stranded) DNA.

The membrane integrity, acrosome, DNA and mitochondrial functional integrity analyses were performed using a WU filter, with excitations of 450–490nm and emission of 516–617nm, by employing an epifluorescence microscope (Olympus BX 51, São Paulo, SP).

Since the Shapiro-Wilk test indicated a lack of normality, all responses were subjected to arcsine transformation. Subsequently, transformed data were compared among treatments using the analysis of variance (ANOVA) with repeated measures, with the effect of individual animals nested within the effect of sperm collections. The comparison of means was done using Tukey’s test. To allow for a straightforward interpretation, the results were reported in their original scale. All analyses were carried out using Statistix™ (2013).

RESULTS

When sperm motility was evaluated in the presence and absence of xanthan gum, a significant difference was observed between the control (no xanthan gum) and xanthan gum treatment (0.25%). At the 24h cooling period, we observed that xanthan gum, at 0.25%, significantly reduced sperm motility, when compared to control values (Table 1).

The mitochondrial functionality measurements (Table 2) revealed no overall difference between control and xanthan gum treatments. However, when the cooling period was considered, the xanthan gum treatments lowered mitochondrial functionality, both after 48h and 72h of cooling. This was not seen when xanthan gum concentration was 0.12% or 0.25%.

Similarly, xanthan gum decreased the percentage of membrane integrity (Table 3). Xanthan gum treatments, as well the control group, reduced membrane integrity as early as 24h of cooling. The acrosome integrity measurements also indicated a significant decline, starting from 24h of cooling (Table 4), both in the control and 0.25% xanthan gum groups.

The DNA integrity was unchanged at different cooling periods, and the statistical analysis revealed no difference between the different groups (Table 5).

DISCUSSION

This is the first study that tested xanthan gum as an additive to the standard diluent used for equine semen refrigeration. Xanthan gum is known to increase the viscosity of the medium, in part, owing to its thickening property in aqueous solution. An increased viscosity due to xanthan gum has been attributed to intermolecular or entanglement interactions, which increase the effective macromolecule size and molecular weight (Garcia-Ochoa *et al*., 2000).

Its higher viscosity prevented cell sedimentation by keeping them dispersed in the diluent medium, being already described in other species as a favorable condition to the cells with the usage of other extenders with higher viscosity, since it eliminates the need for sample homogenization and by promoting a greater contact of the cells with the diluent medium.

We used for this work a slow cooling curve till it reached 5ºC for a 72h storage, non-higher than - 0,05°C/min, between 19 and 8ºC, since in this temperature range the membrane lipids are crossing the transition phase, from a fluid to a gel state, increasing the occurrence of lesions at the level of cell membranes (Squires *et al*., 1999), thereby minimizing the existence

of damages to the plasma membrane by heat shock. This may be testified with the treatment control, in which at the 24h time period it was demonstrated that the membrane integrity rates were equal to the 0h time.

We also observed reduced sperm motility due to xanthan gum, which can be attributed to the increased viscosity at higher concentrations (0.25%) of xanthan gum, potentially hindering the exchanges between the internal medium and the external cellular environment.

Further, the increased viscosity due to xanthan gum may have hampered the movement of the flagellum, impeding sperm cell locomotion, even though mitochondrial functionality was largely unaffected. Previously, (Hu *et al.*, 2009) reported that high concentrations of *Gynostemma pentaphyllum* polysaccharide were associated with decreased sperm motility, membrane and acrosome integrity, and post-thaw mitochondrial activity. In contrast, (Corcini *et al.*, 2011) demonstrated that addition of 1.5% gelatin (thickening medium) in the diluent for cooled porcine semen, maintained sperm quality up to 96h, with lower seminal reflux after insemination.

The acrosome integrity measurements in the current study indicated that although high xanthan gum concentrations (0.25%) were detrimental to acrosome integrity, lower concentrations (0.01% and 0.12%) maintained acrosome integrity up to 24h. No sperm sedimentation occurred at these concentrations when compared to the control diluent, and the samples were well distributed this observation was carried out visually, in which we did not observed cellular sedimentation at the bottom of the tube where xantham gum was added. A homogeneous distribution of cells might have provided an additional layer of protection, shielding these types of structures from deleterious changes caused by cooling. On the other hand, the diluent in the liquid form led to sedimentation in this region of the storage vessel leading to increased pH as result of high concentration of toxic byproducts from cellular metabolism, thereby further accelerating the cellular damage. In studies carried out by Gastal *et al.*, 2017, demonstrated that when production increased by 0.20% and 0.25%, xanthan gum acted efficiently as antioxidant, since post- thawing ROS production of sperm was reduced.

In certain other species of mammals, solid cooling media have demonstrated satisfactory results for semen storage, for periods longer than 24h at temperatures in the range of 5–15°C, (Yaniz *et al.*, 2005; Salvador *et al.*, 2006; Corcini *et al.*, 2011). However, others (Santos *et al.*, 2015) could not verify these results in equine semen, under the same *in vitro* parameters analyzed in our study, with, addition of gelatin to the standard diluents in seminal refrigeration at 5°C did not yield expected benefits. In humans, the higher viscosity of the seminal fluid has been associated with lower sperm motility, leading to infertility (Suarez and

Dai, 1992). The hyperviscosity of the medium above physiological levels has been shown to be detrimental to the viability and stability of the sperm membrane, besides affecting the locomotion and oocyte penetration capacity (Coy *et al.*, 2009). We agree with these interpretations considering the results of the current study using xanthan gum, when we consider *in vitro* aspects for semen quality, in diluents where milk was added and at a temperature of 5ºC, for equine species.

CONCLUSION

Based on the results obtained in this study, we conclude that xanthan gum did not present malicious effects to the sperm cell at concentrations of 0.01 and 0.12% maintaining the seminal characteristics during the cooling similar to the control, while the concentration of 0.25% reduced the seminal quality in the parameter of sperm motility. As a beneficial effect, we can highlight the non-occurrence of cellular sedimentation, not requiring homogenization in the 72 hours of cooling.

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Table list

Table 1. Percentage sperm motility (mean ± standard error of the mean) of stallion semen subjected to cooled storage at 5ºC for 72h and treatment with xanthan gum

Hour	0%	0.01%	0.12%	0.25%
0	85.0 ±1.0^{A,a}	85.0 ±1.0^{A,a}	85.0 ±1.0^{A,a}	85.0 ±1.0^{A,a}
24	60.5 ±1.6^{AB,b}	63.6 ±2.5^{A,a}	51.5 ±2.0^{BC,b}	44.7 ±2.6^{C,b}
48	43.6 ±2.6^{A,bc}	41.0 ±2.1^{A,b}	33.6 ±2.4^{AB,bc}	25.2 ±2.2^{B,bc}
72	26.8 ±3.0^{A,c}	23.1 ±2.9^{AB,b}	18.2 ±2.5^{AB,c}	23.3 ±2.4^{B,c}

^{A,B} Different capital letters indicate the difference in rows (P< 0.05).

^{a,b,c} Different lowercase letters indicate the difference in column

Table 2. Percentage mitochondrial functionality (mean ± standard error of the mean) of stallion semen subjected to cooled storage at 5ºC for 72h and treatment with xanthan gum

Hour	0%	0.01%	0.12%	0.25%
0	76.6 ±1.5^{A,a}	76.6 ±1.5^{A,a}	76.6 ±1.5^{A,a}	76.6 ±1.5^{A,a}
24	74.6 ±2.5^{A,a}	70.9 ±3.9^{A,ab}	69. 7 ±3.2^{A,ab}	70.3 ±3.2^{A,ab}
48	63.0 ±3.6^{A,ab}	63.1 ±2.4^{A,bc}	64.7 ±3.4^{A,ab}	60.0 ±2.5^{A,bc}
72	50.4 ±3.1^{A,b}	50.8 ±4.1^{A,c}	51.8 ±4.9^{A,b}	43.6 ±5.0^{A,c}

^{A,B} Different capital letters indicate the difference in rows (P< 0.05).

^{a,b,c} Different lowercase letters indicate the difference in columns (P< 0.05).

Table 3. Percentage plasma membrane integrity (mean ± standard error of the mean) of stallion semen subjected to cooled storage at 5ºC for 72h and treatment with xanthan gum

Hour	0%	0.01%	0.12%	0.25%
0	81.8 ±1.3^{A,a}	81.8 ±1.3^{A,a}	81.8 ±1.3^{A,a}	81.8 ±1.3^{A,a}
24	61.2 ±1.6^{A,b}	62.3 ±3.2^{A,b}	61.7 ±3.4^{A,b}	62.4 ±2.8^{A,b}
48	43.3 ±4.1^{A,bc}	46.3 ±3.7^{A,bc}	52.9 ±3.3^{A,bc}	51.1 ±2.9^{A,bc}
72	39.4 ±3.3^{A,c}	40.3 ±3.1^{A,c}	43.0 ±3.6^{A,c}	39.6 ±3.0^{A,c}

^{A,B} Different capital letters indicate the difference in rows (P< 0.05).

^{a,b,c} Different lowercase letters indicate the difference in columns (P< 0.05).

Table 4. Percentage acrosome integrity (mean ± standard error of the mean) of stallion semen subjected to cooled storage at 5ºC for 72h and treatment with xanthan gum

Hour	0%	0.01%	0.12%	0.25%
0	79.1 ±1.6^{A,a}	79.1 ±1.6^{A,a}	79.1 ±1.6^{A,a}	79.1 ±1.6^{A,a}
24	61.0 ±2.9^{A,b}	63.6 ±3.2^{A,ab}	63.6 ±3^{A,ab}	62.5 ±3.6^{A,b}
48	55.5 ±2.2^{A,b}	54.1 ±3.2^{A,b}	48.8 ±3.8^{A,b}	58.5 ±3.8^{A,b}
72	52.9 ±4.1^{A,b}	53.5 ±2.9^{A,b}	48.7 ±3.5^{A,b}	48.3 ±3.4^{A,b}

^{A,B} Different capital letters indicate the difference in rows (P< 0.05).

^{a,b,c} Different lowercase letters indicate the difference in columns (P< 0.05).

Table 5. Percentage DNA integrity (mean ± standard error of the mean) of stallion semen subjected to cooled storage at 5ºC for 72h and treatment with xanthan gum

Hour	0%	0.01%	0.12%	0.25%
0	99.8 ±0.5^{A,a}	99.8 ±0.5^{A,a}	99.8 ±0.5^{A,a}	99.8 ±0.5^{A,a}
24	99.8 ±0.5^{A,a}	99.6 ±0.5^{A,a}	98.0 ±1.3^{A,a}	98.8 ±1.2^{A,a}
48	99.4 ±0.5^{A,a}	98.4 ±0.6^{A,a}	97.7 ±1.6^{A,a}	99.6 ±0.1^{A,a}
72	99.3 ±0.5^{A,a}	99.4 ±0.5^{A,a}	97.7 ±2.1^{A,a}	99.6 ±0.5^{A,a}

^{A,B} Different capital letters indicate the difference in rows (P< 0.05).

^{a,b,c} Different lowercase letters indicate the difference in columns (P< 0.05).

3.2 Artigo 2

Semen Cryopreservation of the Amazonian Catfish (*L. marmoratus*) with Dimethyl Sulfoxide as Cryoprotectant

Stela Mari Meneghello Gheller, Carine Dahl Corcini, Geórgia da Cruz Tavares, Edenara Anastásio da Silva, Izani Bonel Acosta, Camila Ribeiro Carvalho de Brito, Alessandra Cardoso Silva, Antonio Sergio Varela Junior

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SEMEN CRYOPRESERVATION OF THE AMAZONIAN CATFISH (*LEIARIUS MARMORATUS)* WITH DIMETHYL SULFOXIDE AS CRYOPROTECTANT

GHELLER, Stela Mari Meneghello[1]; CORCINI, Carine Dahl[1,2;] TAVARES, Geórgia da Cruz[1]; SILVA, Edenara Anastásio[1]; ACOSTA, Izani Bonel[1]; BRITO, Camila Ribeiro Carvalho[1;] SILVA, Alessandra Cardoso[1]; VARELA JUNIOR, Antonio Sergio [1,2;]

[1]*Grupo de pesquisa ReproPel - Faculdade de Veterinária – UFPel*
[2] *RAC, Reprodução Animal Comparada, Instituto de Ciências Biológicas, Universidade Federal de Rio Grande (FURG).*

Author address (for correspondence): Antonio Sergio Varela Junior, Universidade Federal de Rio Grande, Rio Grande/ RS, Campus Universitário s/n – Campus Carreros, Prédio 06, Instituto de Ciências Biológicas ICB – Histologia, Vila Maria CEP: 96203000 – Rio Grande, RS, Brasil. E-mail: varelajras@hotmail.com

Abstract

The current study followed the elaboration of a protocol of semen cryopreservation and assessed the sperm motility and integrity parameters of the Amazonian catfish *Leiarius marmoratus*, a freshwater fish, from Siluriformes Order, acknowledged for being extremely important for Brazilian fish farming. Eight males were hand-stripped and semen was frozen using the internal cryoprotectant dimethyl sulfoxide (DMSO) at the following concentrations: 5, 10, 15, and 20%. Semen was diluted in Beltsville Thawing Sollution (BTS). Samples were diluted in tested media (1:9, v/v), frozen in dry-shipper canister for 12 hours at -60°C, and stored in liquid nitrogen at –196°C. Kinematic (sperm motility and sperm duration) and morphological (membrane and DNA integrity, and mitochondrial function) semen parameters were assessed post-thawing under fluorescence microscopy. Regardless the DMSO concentrations tested, no statistical differences were observed in the following assessments: membrane integrity, DNA integrity, mitochondrial function, sperm motility, and motility duration ($P > 0.05$). When total and progressive motilities, Velocity curved line (VCL), Velocity straight line (VSL) and Linearity were assessed, treatment containing 5% of DMSO displayed inferior results when compared to treatments containing 15 and 20% DMSO ($P < 0.05$). Treatment containing 10% DMSO did not differ from other treatments ($P > 0.05$). The

freezing protocol tested in the current study provides a promising method and enables the use of this biotechnology for animal breeding programs in fish farms and the maintenance of the genetic variability. Thus, the optimal DMSO concentrations for freezing are 15% and 20%, diluted in BTS extender, at the freezing rate used for *L. marmoratus*.

Keywords: cryopreservation protocol, dimethyl sulfoxide, Siluriformes fish.

Introduction

The Amazonian catfish *L. marmoratus* is a freshwater fish of Siluriformes Order, Family Pimelodidae, found in the Amazon river basin, currently being affected in its natural habitat by environmental changes caused by human activities such as the construction of hydroelectric dams, changes in river courses, in addition to overfishing (Viveiros et al., 2009).

Currently, in an attempt to reduce the cannibalism in the initial phases, fish farms breed male broodstock of *L. marmoratus* with female broodstock of *Pseudoplatystoma corruscans*, resulting in hybrids offspring (Galo et al., 2014; Mateo et al., 2008). However, the breeding season of these two species does not occur synchronously, therefore requiring the development of semen cryopreservation protocols to optimize the breeding and hybrid offspring production. Hitherto, no semen cryopreservation protocol has been described for *L. mamoratus*, though a cooling protocol that sets the semen samples at 13°C (± 2°C) has been assessed for the following parameters: total and progressive motilities, motility duration, and sperm morphology (Galo et al., 2014).

The combination extender/cryoprotectant (Beltsville Thawing Solution [BTS]/Dimethyl Sulfoxide [DMSO]) is widely used to freeze the sperm of freshwater fish: *Salminus brasiliensis* (Viveiros et al., 2009), *Brycon orbigymus* and *Prochilodus lineatus* (Viveiros et al., 2015), *Colossoma macropomum* (Garcia et al., 2015), and the freezing rate describe by Taitson et al. (2008). Dimethyl sulfoxide acts by interacting or combining with nucleic acids, carbohydrates, lipids, and proteins without irreversibly changing the molecular configuration of these compounds due to the rapid penetration in the cell (Sojka et al., 1990). Yet, DMSO also interacts with structural phospholipids in the cell membrane, maintaining the water exchange at 0°C, reducing ice crystal formation by decreasing the freezing time of the intracellular fluid during the cryopreservation process (Thirumala et al., 2006).

The extender BTS stands out for its high-content of sugar, that provides energy to the sperm cells, and the presence of ions in the freezing extender that control the osmotic pressure

during cell dehydration process (Kopeika and Kopeika, 2008). Thus, the microenvironment provided is osmotically favored and sources the inner side of plasma membrane with protection (Murgas et al., 2007).

In view of the above, this study tested a semen cryopreservation protocol for the Amazonian catfish (*L. marmoratus*) semen, by associating the BTS extender and different concentrations of DMSO (5, 10, 15, and 20%), and submitting the samples to a freezing rate using a dry-shipper container. Then, sperm parameters were assessed post-thawing: membrane and DNA integrities, mitochondria functionality, total motility, and motility duration.

Materials and Methods

All reagents used in this experiment were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Semen collection, sperm cryopreservation, and extenders

The broodstock were obtained (n=8) from a commercial fish farm (Pimenta Bueno, Rondonia, Brazil - 11°41'46.95"S e 61°13'47.50"W), during breeding season (November/December). Semen collection was performed through hand stripping 6.5h (175 degree-hours) after hormonal induction (carp pituitary extract – 1mg/kg) (BILLARD; COSSON and CRIM, 1995).

Sperm activation with urine, feces, or water was investigated prior to total motility assessments and, if so, samples were discarded. Otherwise, semen samples were activated with activation solution (199 mM $NaHCO_3$) and, after 10s, the motility rate was recorded. Only samples which the motility rate was higher than 80% were selected for cryopreservation. Assessments were conducted by observing semen samples (5 µL) loaded on a glass slide through an optical microscope (Olympus CHK2-F-GS, América INC, São Paulo, SP) (CAROLSFELD et al., 2003).

Samples were diluted at 1:9 rate (semen:extender) in Beltsville Thawing Solution (extender base solution) (PURSEL, V. G. and JOHNSON, L. A., 1975) and DMSO was added at 5%, 10%, 15%, and 20% concentrations. Samples were loaded into 0.25 mL straws and stabilized for 10 minutes at room temperature (~24°C) (VARELA JUNIOR, A. S. et al., 2012). Then, samples were stored in dryshipper canister (Taylor-Wharton, model CP 300 *dry shipper*) at -60°C for 12h, at a freezing rate of -28°C/min, and thus transferred, and stored in liquid nitrogen canister at -196°C.

In vitro *analyses of sperm quality post-thawing*

Straws were submerged in water-bath at 45°C for 8 seconds and 0.40 mL of BTS extender at 22°C was added to the thawed samples (VARELA JUNIOR, A. S. et al., 2012). Sperm analysis was performed thereafter.

a) Sperm motility and Sperm duration

Sperm was activated with activation solution (NaHCO3, 119 mM, 1:4 semen:solution) and loaded on a slide covered with coverslip. Samples were assessed by CASA-system (SpermVision®, Minitube, Tiefenbach, Germany), coupled to an optical microscope (Axio Scope A1®, Zeiss, Jena, Germany), and observed at 200 X magnification. Variables assessed by CASA were Velocity average path (VAP), Velocity curved line (VCL), Velocity straight line (VSL), Linearity (LIN), Total motility (TMO), and Progressive motility (PMO). For each sample, at least 500 cells were counted along 10 observation fields captured. In the sperm duration, shortly after semen was diluted into an activation solution (119 mM of $NaHCO_3$) a chronometer was triggered and then stopped when sperm flagellum ceased movement within the optical field (VARELA JUNIOR, A. S. et al., 2012).

b) Cell integrity analyses

Membrane integrity, DNA integrity, and mitochondria functionality were assessed in an epifluorescence microscope at 400X magnification (Olympus BX 51, América INC, São Paulo, SP), using WU filter (450-490 nm excitation and 516-617 emission). Mitochondria functionality was assessed with rhodamine 123 fluorescent probe (13µM), after incubation at 20°C for 10 minutes (VARELA JUNIOR, A. S. et al., 2012). Membrane integrity was assessed adding to the sperm cells the fluorescent probes carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) (HARRISON, R. A. P. and VICKERS, S. E., 1990). DNA integrity was assessed adding to the sperm cells acridine orange probe (BENCHARIF, D. et al., 2010).

Statistical analysis

Dependent variables were tested for normality using Shapiro-Wilk's test. Then, an Analysis of Variance (ANOVA) with means comparison through Tukey's test was performed. Different cryoprotectants were considered as independent variables and sperm motility, motility duration, mitochondria functionality, DNA integrity, and membrane integrity were considered as dependent variables. All data were expressed as Means ± Standard Error Means (SEM). All analyses were made using Statistix 9.0 (2010) software.

Results

The average values for fresh semen were 4.5 mL (± 0.3 mL) of volume, 8.7×10^9 sperm cells/mL (± 0.2×10^9 sperm cells/mL) of sperm concentration, 95.7 % (± 2.0 %) of sperm motility, and 122.6 seconds (± 5.0 seconds) of motility duration.

Analyses performed post-thawing showed that there were non-significant differences between treatments, regardless the concentrations of DMSO tested for membrane integrity, DNA integrity, mitochondria functionality (Table 1), and motility duration (Table 2) assessments.

For total motility analysis, the treatment containing DMSO at 5% (12.9%) was inferior to treatments with DMSO at 15% and 20% (24.8% and 30.2%, respectively). Treatment containing DMSO at 10% did not differ from other treatments (15.8%). Progressive motility exhibited same tendency as in total motility: treatment with DMSO at 5% (9.5%) was inferior to treatments with DMSO at 15% and 20% (18.6% and 24.0%, respectively). Treatment with DMSO at 10% did not differ from the other treatments (15.8%). As for motility variables assessed by CASA-system VCL, VSL, and LIN same pattern of analysis was observed with respect to the cryoprotectant concentrations (Table 2).

Discussion

Even before the success of different cryopreservation protocols for semen of native freshwater fish, until the present study the association of DMSO and BTS has not yet been described for *L. marmoratus.* Likewise, it was unknown cryoprotectant concentration, extender and freezing rates suitable for this species, which currently represents great commercial

importance in the Brazilian fish farming. Therefore, this study depicts a pioneering endeavor to cryopreserve the semen of this Amazonian catfish.

In the post-thawing *in vitro* analyses, the parameters motility duration and DNA integrity did not seem to have undergone interferences despite of the DMSO concentrations tested, exhibiting satisfactory results when analyzed singly. As for motility duration, previous studies demonstrated that results are adequate when samples were activated with activation solution after cryopreservation with DMSO (CAROLSFELD et al., 2003). In the current study, motility duration post-thawing was higher than 60 seconds in all treatments tested, which can be considered a satisfactory result since during the fertilization process the oocyte micropyle remains open for less than one minute, after its activation when in contact with water.

The results in DNA integrity were higher than 80% in all treatments tested, which might suggest that DMSO prevented the denaturation of this structure during the freezing-thawing process. This result corroborates one obtained by GARCIA et al. (2015), in which the association extender/cryoprotectant achieved results higher than 80% for this parameter in other freshwater fish species. However, membrane integrity rates were less than 45%, regardless DMSO concentration tested. In this study, we used a stabilization time of 10 minutes (~25°C) before the beginning of the freezing rate. DMSO when in contact with cell structures at room temperature may cause denaturation to membrane proteins and, therefore, damage the plasma membrane due to its toxicity and the osmotic shock.

Part of the damages occasioned to the cell membrane and consequently to the mitochondria and a diminished sperm motility might have been caused by the absence of an external cryoprotectant that could aid stabilize the cell membrane during the critical phase in the freezing process, since we only used an internal cryoprotectant added to the extender. Some authors state that non-penetrating cryoprotectants, such as disaccharides, protect the cell membrane against the alteration in cell volume during freezing-thawing process (SILVA et al., 2015).

Given the results obtained in this study regarding sperm motility and cell integrity, we suggest the addition of an external cryoprotectant, such as trehalose (ACOSTA-SALMON et al., 2007; GHELLER et al., 2019) or egg yolk (NINHAUS-SILVEIRA et al., 2006), in an attempt to reduce structural damages caused by freezing process, considering that we obtained a relative success in preserving the DNA integrity and the motility duration with the use of DMSO.

The diluent and freezing protocol tested in current study provided a promising approach, considering that this was the first step to stablish a cryopreservation protocol for this species,

enabling this technique to be used in animal breeding programs in fish farms aiding the preservation of genetic diversity. Thus, this may constitute an alternative for gametes availability during breeding season, since this species is of great importance commercially for fish farming in Brazil.

Conclusion

This study demonstrated that semen samples from Amazonian catfish *Leiarius marmoratus* could undergo cryopreservation process using DMSO at 15% and 20% concentration in BTS extender at the freezing rate proposed, which maintain semen parameters post-thawing.

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Table list

Table 1. Effect of different concentrations of DMSO (5, 10, 15, and 20%) on cryopreserved semen samples of *L. marmoratus*, on sperm quality parameters membrane integrity, DNA integrity, and Mitochondria functionality (means ± standard error means) (n=8).

Treatments	Membrane Integrity (%)	DNA integrity (%)	Mitochondria functionality (%)
DMSO 5%	33.2 ± 4.8	83.5 ± 6.2	52.2 ± 5.2
DMSO10%	28.0 ± 3.1	84.5 ± 6.2	46.0 ± 5.7
DMSO 15%	31.5 ± 5.0	82.5 ± 8.4	50.2 ± 8.1
DMSO 20%	42.0 ± 6.7	80.0 ± 9.4	66.0 ± 2.0

Data are expressed as of intact sperm cells in relation to total cell counting (%).

There is no significant diferences between treatments (P<0.05).

Table 2. Effect of different concentrations of DMSO (5, 10, 15, and 20%) on cryopreserved semen samples of *L. marmoratus*, on sperm motility parameters assessed by CASA-system: Total Motility (TM), Progressive motility (PM), Motility Duration (MD), Velocity average path (VAP), Velocity curved line (VCL), Velocity straight line (VSL), Linearity (LIN) (means ± standard error means) (n=8).

	DMSO 5%	DMSO 10%	DMSO 15%	DMSO 20%
LIN	0.4 ± 0.05^{B}	0.66 ± 0.02^{A}	0.7 ± 0.02^{A}	0.6 ± 0.06^{A}
VAP (µm/s)	19.3 ± 2.7^{B}	25.9 ± 1.8^{AB}	31.7 ± 2.1^{A}	28.2 ± 3.5^{AB}
VCL (µm/s)	24.6 ± 3.4^{B}	33.2 ± 2.2^{AB}	38.0 ± 2.0^{A}	32.1 ± 3.9^{A}
VSL (µm/s)	15.6 ± 2.3^{B}	22.3 ± 1.7^{AB}	28.5 ± 2.2^{A}	25.7 ± 3.3^{A}
TM (%)	12.9 ± 2.1^{B}	21.5 ± 1.5^{AB}	24.8 ± 2.2^{A}	30.2 ± 3.2^{A}
PM (%)	9.5 ± 2.2^{B}	15.8 ± 1.5^{AB}	18.6 ± 2.1^{A}	24.0 ± 3.0^{A}
MD (s)	70.0 ± 22.6	105.0 ± 23.6	60.0 ± 12.8	110.0 ± 44.1

Distinct letters indicate statistical differences within rows (P<0.05).

3.3 Artigo 3

Use of trehalose in the semen cryopreservation of Amazonian catfish *Leiarius marmoratus*

Stela Mari Meneghello Gheller, Carine Dahl Corcini, Camila Ribeiro Carvalho de Brito Izani Bonel Acosta, Geórgia da Cruz Tavares, Sara Lorandi Soares, Alessandra Cardoso Silva, Diego Martins Pires, Antonio Sergio Varela Junior

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Use of trehalose in the semen cryopreservation of Amazonian catfish *Leiarius marmoratus*

Stela Mari M Gheller[1]; Carine D Corcini [1,2]; Camila R C de Brito[1]; Izani B Acosta[1]; Geórgia C Tavares[1]; Sara Lorandi Soares[1]; Alessandra C Silva[1]; Diego M Pires[1]; Antonio Sergio Varela Junior [1,2];

[1]ReproPel Research Group – Veterinary Faculty – Universidade Federal de Pelotas (UFPel)

[2] RAC, Reprodução Animal Comparada, Instituto de Ciências Biológicas, Universidade Federal de Rio Grande (FURG).

Author address (for correspondence): Antonio Sergio Varela Junior, Universidade Federal de Rio Grande, Rio Grande/ RS, Campus Universitário s/n – Campus Carreros, Predio 06, Instituto de Ciencias Biologicas ICB – Histologia, Vila Maria CEP: 96203000 – Rio Grande, RS, Brasil. E-mail: varelajras@hotmail.com

Abstract

The current study assessed a semen cryopreservation protocol in the Amazonian catfish *Leiarius marmoratus*, a freshwater fish, of rheophilic behavior, and of great importance for Brazilian fish farming. Eight males (n=8) were stripped and the semen was cryopreserved if total motility in fresh semen was higher than 80%. The external cryoprotectant Trehalose was then diluted in Beltsvile Thawing Solution (BTS) extender in the following concentrations: 50, 100, 150, and 200 mM. Semen samples were diluted in the media (1:9 v/v) being tested, then frozen in a container with nitrogen vapor (dryshipper), and stored in liquid nitrogen at -196°C. Motility parameters assessed post-thawing were performed by CASA-system and sperm cell integrity analyses (membrane integrity, DNA integrity, and mitochondrial function) were

performed through fluorescence microscopy. As a result, no significant statistical difference was observed between treatments, independently of Trehalose concentrations tested in the following post-thawing analysis: membrane integrity, DNA integrity, mitochondrial functionality, and sperm motility duration. As of total and progressive motilities, the treatment containing 50 mM trehalose (15.6 and 9.5%, respectively), exhibited inferior results when compared to treatments with 150 mM (22.9 and 17.7%, respectively) and 200 mM (31.4 and 26.3%, respectively) trehalose concentrations($P < 0.05$); however, it did not differ from the treatment with 100 mM trehalose (18.6 and 15.3%, respectively). Therefore, treatments with trehalose at higher concentrations exhibited superior results when compared to other treatments in *in vitro* motility parameters for *L. marmoratus*.

Keywords: External cryoprotectant, *in vitro* analysis, freshwater fish, sugar

Introduction

Sperm cryopreservation is a biotechnique that guarantees constant supply of semen and the conservation of genetic diversity (CABRITA et al., 2010). Although cryopreservation has benefited fish production, current studies aim to decrease or eliminate the aspects that reduce spermatozoon viability and fertility post-thawing (STREIT JR et al., 2006) in different fish species used in fish farming due to individual differences among them (SALMITO-VANDERLEY et al., 2012).

Amazonian catfish *Leiarius marmoratus* is a freshwater fish with natural rheophilic behavior, which has been enduring anthropogenic environmental disturbances. Highly reared in Brazilian fish farming, its gamete production only occurs after hormonal induction. Even with the commercial importance of *L. marmoratus*, it lacks a cryopreservation protocol that has to be established.

In this context, the search for standardization of sperm cryopreservation in this species may aid decrease sperm quality losses with the use of external cryoprotectants, since they act directly on sperm organelles (HOLT, 2000). The best examples are carbohydrates, which may act as cryoprotectant agents depending on their molecular weight (HOLT, 2000). Trehalose is a disaccharide consisting of two glucose molecules and is one example of sugar that assists cell dehydration due to its hyperosmolarity. It helps reduce the formation of intracellular ice crystals, thus decreasing cryoinjuries resulting from freezing processes (AISEN et al., 2005). Moreover, it has antioxidant capacity that protects the membrane against free radical attacks – reactive oxygen species (ROS) (BUCAK et al., 2007).

Studies have shown that only the use of trehalose is able to maintain semen quality rates post-thawing without the addition of internal cryoprotectant and in pigs, these results were achieved with final concentration of 100 mM trehalose (ATHURUPANA et al., 2015), the same

was described in tambaqui milt with 150 mM trehalose (VARELA JUNIOR, A. et al., 2012), and in *Pagrus major* with 100 mM trehalose (LIU et al., 2015).

With the above, the objective of this study was to assess the effect of different concentrations of trehalose on *in vitro* parameters of semen quality post-thawing of *L. marmoratus*.

Materials and Methods

All reagents used in this experiment were purchased from Sigma Aldrich® (St. Louis, MO, USA).

Male broodstock

Males were provided by Boa Esperança Fish Farm (11°41'46.95"S and 61°13'47.50"O), at the municipality of Bueno/RO/Brazil, during spawning season (November to February), with the temperature of water tanks set to 26°C. Initially, ten males were hormonally induced with carp pituitary extract (1 mg/kg live animal), diluted in 0.5 mL of sterile saline (0.9% NaCl), and injected into the dorsal muscle of each fish. After 6.5 h, fish were stripped for semen collection through abdominal massage, following the recommendations of BILLARD; COSSON; PERCHEC; et al. (1995). In two of the ten semen samples collected there was contamination with urine and water and they had to be discarded. Therefore, the semen samples of males used in this experiment were eight in total (n=8).

Fresh semen analysis and freezing protocol

The analysis of motility of fresh semen samples were expressed subjectively through optical microscopy shortly after semen collection and was performed in a laboratory adjacent to the area of the fish tanks. To avoid loss of viability, the samples that were collected and not

immediately assessed were kept in a cooled box at 5°C while a previously collected sample was being assessed in the microscope. It consisted of the observation of a small drop of fresh semen loaded on a slide and covered with coverslip 10 seconds after activation with distilled water, using an optical microscope (Olympus CHK2-F-GS, América INC, São Paulo, SP) at 200X magnification. Samples were selected if motility rates were equal to or higher than 80%, otherwise they were discarded.

Samples selected were then diluted at a rate of 1:9 (semen:extender), using as base solution the extender Beltsville Thawing Solution (BTS) (PURSEL, V. and JOHNSON, L., 1975) and the different concentrations of trehalose tested (50 mM, 100 mM, 150 mM, and 200 mM) (VARELA JUNIOR, A. et al., 2012). BTS extender demonstrates efficiency in maintaining the viability of sperm cells of freshwater fish due to its adequate osmolarity and pH (VARELA JUNIOR, A. et al., 2012; VIVEIROS et al., 2009). Soon afterwards, samples were stored in 250 µL straws (2 straws/treatment) and kept in dry shipper cylinder (Taylor-Warton, CP 300 model dry shipper) for 24h (at -60°C, and freezing rate of approximately -28ºC/min) (TAITSON et al., 2008). Then, they were transferred and stored into liquid nitrogen canister (at -196°C), where they remained until thawing.

In vitro *analyses of sperm quality post-thawing*

All analyses performed post-thawing were conducted at ReproPEL Andrology Laboratory (Pelotas, Rio Grande do Sul, Brazil), where CASA-system was located and distant 3,358.0 km from the fish farm. Straws containing semen samples of each male were carefully removed from the nitrogen canister and placed into water bath at 45°C for 5 seconds (STREIT JR et al., 2006) and 400 µL of BTS extender (at 22°C) was added to the final volume of the semen thawed to decrease cryoprotectant toxicity. Then, analysis of motility rate (total and progressive, assessed by CASA-system), motility duration, membrane integrity, DNA integrity,

and mitochondria functionality tests were performed. For cell integrity analyses (membrane integrity, DNA integrity, and mitochondria functionality), assessments were made by observing sperm cells in an epifluorescence microscope (Olympus BX 51, America INC, São Paulo, SP), at 400X magnification, with WU filter 450-490 nm excitation and 516-610 nm emission, after incubating samples for 5 minute at ~22°C. Data were expressed in percentage (%) as of intact sperm cells in relation to total cell counting (Table 1) and for each parameter we counted 200 sperm cells.

a) Sperm motility

Samples were assessed by CASA-system (SpermVision®, Minitube, Tiefenbach, Germany), coupled to an optical microscope (Axio Scope A1®, Zeiss, Jena, Germany), and observed at 200 X magnification. Sperm was activated with activation solution, consisted of a sodium bicarbonate solution ($NaHCO_3$, 119 mM, 1:4 semen:solution) and loaded on a slide covered with coverslip. Variables assessed by CASA were: Velocity average path (VAP), Velocity curved line (VCL), Velocity straight line (VSL), Linearity (LIN), Total motility (TMO), Progressive motility (PMO). For each sample, at least 500 cells were counted along 10 observation fields captured.

b) Motility duration

Sample aliquots of 1 µL semen were homogenized with 4 µL of activation solution (same solution used in the sperm motility analysis) and loaded on slides covered with coverslip. A timer was triggered and the duration of sperm motility was recorded until sperm cells ceased moving.

c)Plasma membrane integrity

An aliquot of 10 µL of semen sample was diluted in 40 µL of isotonic saline solution containing 1.7 mM formaldehyde, 20 M carboxyfluoresceine diacetate (CFDA), and 7.3 µM propidium iodide (PI) –, then incubated for 5 minutes at room temperature (approximately 22°C) in a dark room (HARRISON, R. and VICKERS, S. E., 1990). Cells exhibiting green fluorescence were considered intact, that means CFDA remained within cell cytoplasm. Otherwise – red or greenish-red fluorescence –, cells were considered damaged, since the intact membrane is not permeable to PI.

d)DNA integrity

For DNA integrity assessment, 45 µL of semen sample was mixed with 50 µL of TNE solution (0.01 M Tris-HCL; 0.15 M NaCl; 0.001 M EDTA, pH 7.2) and let in wait for 30 seconds. Then, 200 µL of Triton solution (1X) was added to the mixture and, 30 seconds later, 50 µL acridine orange was added (2 mg/mL in deionized water). Samples were observed in the microscope after 5 minutes incubating at room temperature (~22°C). Cells exhibiting green fluorescence had the DNA intact, otherwise – red or orange fluorescence –, DNA was considered damaged (BENCHARIF, DJEMIL et al., 2010).

e) Mitochondrial function

The assessment was performed after incubating 10 µL of semen sample mixed with 40 µL rhodamine 123 solution (13 µM), at 20°C for 10 minutes. When rhodamine-positive staining (green fluorescence) was present, sperm cells had functional mitochondria. Non-functional mitochondria exhibited rhodamine-negative staining, that means fluorescent cells were undetected (HE and WOODS, 2004).

Statistical analysis

Normality test was performed for all dependent variables by Shapiro-Wilk test. Afterwards, analysis of variance was performed with means comparison by Tukey test. Different cryoprotectants were considered as independent variables, and the variables sperm motility, motility duration, mitochondrial function, DNA integrity, and plasma membrane integrity were considered as dependent variables. All data were expressed as mean ± standard error means (SEM). All analyses were performed using the software Statistix 9.0 (2010).

Results

From the eight males collected, the average volume and sperm concentration were, respectively, 4.1 ± 0.3 mL and 8.7 ± 0.2 x 10^9/mL. In fresh semen, the motility parameters were estimated by optical microscopy, as previously described and average total motility and motility duration were 95.7 ± 2.0% and 122.6 ± 5.0 seconds, respectively.

Different concentrations of trehalose did not exhibit significant statistical effect on cell integrity parameters: mitochondrial function, DNA integrity, and membrane integrity (Table 1; $P > 0.05$).

As for motility parameters (Table 2), semen samples with trehalose concentrations higher than 50 mM had significantly superior velocity parameters (VAP, VCL, VSL, and LIN; $P < 0.05$), when compared to the samples with the lowest trehalose concentration. Samples containing 200 mM trehalose had TMO and PMO (31.4% ± 1.8 and 26.3% ± 1.8, respectively) significantly superior when compared to other treatments, but significantly lower when compared to fresh semen sample. In the parameter motility duration, treatment with 200 mM trehalose exhibited the best result, though it did not differ from treatment with 50 mM trehalose.

Discussion

The effect of trehalose used for cryopreservation of *L. marmoratus* sperm cells was still unknown and, in this study, it was observed that the concentration of 200 mM demonstrated higher effect on sperm motility rates when compared to the other concentrations tested. The cryoprotectant effect of trehalose is due to its osmolarity and specific interactions with phospholipids in the plasma membrane (BAKAS and DISALVO, 1991; XI et al., 2018), especially with polar groups of phospholipids, preventing lateral spacing between them, inhibiting fusion, juxtaposition, and the rupture of the membrane (ANCHORDOGUY et al., 1987; CROWE et al., 1985).

Studies of molecular modeling suggest that trehalose can protect against water withdrawal during dehydration. It replaces the water of crystallization associated with biological structures that help stabilize these biomolecules and inhibit irreversible dehydration (DONNAMARIA et al., 1994). Moreover, the increase in the osmotic pressure of high molecular weight sugars promotes cell dehydration, and decreases the formation of intracellular ice crystals (NAGASE, 1964). Therefore, the results observed for motility parameters may be explained by the protector effect trehalose had on sperm cells during freezing and thawing processes.

Yet, trehalose also aids reduce the cell damages whereas it binds to membrane phospholipids making the cells more resistant to freezing and thawing processes. It also protects biomolecular structures, either by replacing the hydrogen-bonded water (CHEN et al., 2000), or by promoting the hydration required for cell support post-thawing (PATIST and ZOERB, 2005). Studies on the hydration potential of trehalose, when compared to other oligosaccharides, report that this sugar has a higher capacity for hydration. It suggests that trehalose possibly stabilize lipid bilayers either by ordering water molecules around the

membrane or by interacting directly with polar biomolecules when water is removed (KAWAI et al., 1992).

As of membrane integrity and mitochondrial function, the rates achieved post-thawing differ from the outcomes reached in other studies (LIU et al., 2015; VARELA JUNIOR, A. et al., 2012), in which trehalose concentrations superior to 150 mM demonstrated negative effects on cryopreservation. Different effects may be related to the glycolipid composition of the plasma membrane that varies between species and has a correlation with the degree of susceptibility of the spermatozoa to cooling, freezing, and thawing rates (HOLT, 2000). These differences may be related to the trehalose concentrations tolerated by sperm cells.

As for DNA integrity assessments, we did not observe statistical differences between treatments tested ($P > 0.05$), though the averages of intact cells were higher than 60%. This result is promising, considering that genetic information passed to the next generations is concealed within this structure. It is reported in other studies (CABRITA et al., 2010) that the preservation of genome information must be number one priority in cryopreservation protocols, considering that DNA integrity must be maintained to have successful embryo development after fertilization. Other studies where fertilization was assessed in different fish species, semen cryopreserved with trehalose in the extender composition was able to fertilize the eggs after thawing process, although the fertilization rates were lower when compared to the use of fresh semen (LICHTENSTEIN et al., 2010; MIYAKI et al., 2005; XI et al., 2018).

Aside the cryoprotectant effect, trehalose also has antioxidant effect (LIU et al., 2015). Some researchers suggested that the oxidative damage to mitochondrial DNA and plasma membrane might be an important determinant to explain the fertility and motility of cryopreserved sperm cells. Oxidative stress is associated to an increase of oxidative rate in cell components and the excess of ROS production (AITKEN et al., 1996; ALVAREZ and STOREY, 1992). In fish, some of ROS negative effects on sperm quality have been reported

(LAHNSTEINER and MANSOUR, 2010; SHALIUTINA-KOLEŠOVÁ et al., 2013). It demonstrates the important role that antioxidants play to maintain the sperm motility and genomic integrity (CABRITA et al., 2011). The dilution before cryopreservation decreases the concentration of seminal fluid components that could have antioxidant effect, but also other components as seminal fluid proteins that prolong and stabilize sperm viability (MARTÍNEZ-PÁRAMO et al., 2009).

In other studies conducted on cryopreservation of semen of species related to *L. marmoratus*, it was noted that BTS-DMSO and BTS-Methyl-Glycol associations used to prepare the freezing extenders exhibited similar motile parameters results such as in our study (VIVEIROS et al., 2015).

Sperm cryopreservation provides an important tool for the development of animal breeding programs in fish farming, as well as facilitating the management of artificial reproduction. Although the analyses of fertilization and hatching rate were not performed, the in vitro analyzes conducted displayed promising results in the maintenance of cell integrity and sperm motility parameters. This study therefore provides an interesting protocol to freeze sperm samples of *L. marmoratus* species, to be used for commercial purposes or as a tool to assemble a germplasm bank for this species.

Conclusion

Motility data and cellular integrity parameters showed that trehalose concentrations of 100 – 200 mM have a positive effect on sperm quality during cryopreservation of sperm cells of *L. marmoratus*.

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Table list

Table 1. Effect of trehalose concentrations on membrane integrity, DNA integrity and mitochondria functionality (% of intact sperm cells in relation to total cell counting) in cryopreserved semen samples of Amazonian catfish *L. marmoratus* (means ± standard errors means) (n=8).

Extenders	Integrity		Mitochondria functionality (%)
	Membrane (%)	DNA (%)	
Trehalose 50 mM	36.0 ± 9.4	65.4 ± 9.4	69.1 ± 4.8
Trehalose 100 mM	38.8 ± 1.9	84.2 ± 5.5	67.8 ± 2.3
Trehalose 150 mM	35.2 ± 2.1	60.0 ± 8.8	67.5 ± 2.6
Trehalose 200 mM	42.0 ± 3.5	66.2 ± 10.2	71.5 ± 4.8

There were no statistical differences between treatments in the results exhibited above (comparisons within columns) (P>0.05)

Table 2. Effect of trehalose concentrations on post-thawing sperm quality parameters of sperm motility assessed by Computer-assisted sperm analysis (CASA) and the duration of sperm motility in cryopreserved semen samples of Amazonian catfish *L. marmoratus* (means ± standard errors means) (n=8).

	Trehalose 50mM	Trehalose 100mM	Trehalose 150mM	Trehalose 200mM
LIN	0.40 ± 0.05^{C}	0.60 ± 0.04^{B}	0.68 ± 0.03^{AB}	0.70 ± 0.01^{A}
VAP (µm/s)	17.0 ± 2.2^{C}	27.7 ± 2.1^{B}	30.4 ± 2.2^{AB}	35.9 ± 1.5^{A}
VCL (µm/s)	21.2 ± 2.7^{B}	33.1 ± 2.4^{A}	27.5 ± 2.2^{A}	42.4 ± 1.5^{A}
VSL (µm/s)	15.1 ± 2.0^{B}	25.1 ± 2.0^{A}	27.5 ± 2.2^{A}	32.2 ± 1.5^{A}
TMO (%)	13.6 ± 1.9^{C}	18.6 ± 1.7^{BC}	22.9 ± 1.4^{B}	31.4 ± 1.8^{A}
PMO (%)	9.5 ± 1.6^{C}	15.3 ± 1.4^{BC}	17.7 ± 1.5^{B}	26.3 ± 1.8^{A}
MD (s)	65.7 ± 14.1	51.8 ± 11.5	52.8 ± 11.4	84.2 ± 11.5

Different letters represent statistical differences between treatments (rows) (P<0.05).
Linearity (LIN), Velocity average path (VAP), Velocity curved line (VCL), Velocity straight line (VSL), Total motility (TMO), Progressive motility (PMO), Motility duration (MD).

4 Considerações Finais

De uma forma geral, podemos concluir que os procedimentos de refrigeração seminal e criopreservação para a utilização de técnicas de reprodução artificial é de fundamental importância para o desenvolvimento e crescimento da cadeia produtiva. Estudos visando a melhor eficiência desses processos contribuem para o melhoramento genético dos planteis, potencializando o uso de machos de alto valor reprodutivo, além de facilitar a troca de material genético entre produtores pelas facilidades de transportes dessas doses inseminantes em botijão de nitrogênio e caixas acondicionadoras.

No primeiro estudo referente ao uso da xantana adicionada ao diluente kenney no sêmen equino, os resultados demonstraram que a mesma não promove efeito tóxico para a célula, além de manter as células dispostas no meio sem ocorrência de sedimentação durante o período de refrigeração. Nos resultados encontrados na condição experimental testada não se evidenciou uma melhora nas estruturas da célula espermática refrigerada, e na concentração mais elevada (0,25%) o mesmo dificultou o deslocamento da célula no campo analisado ao microscópio, pela espessura do meio que não se desfez posterior o aquecimento (37°C) para análise de motilidade seminal pós refrigeração.

No segundo estudo com a utilização de dimetilsulfóxido na criopreservação de células espermáticas de *Leiarius marmoratus*, mesmo esse sendo o crioprotetor interno mais empregado para a criopreservação em peixes de água doce, nessa espécie com as concentrações testadas, meio diluente BTS e curva de congelamento, não se obteve resultados esperados quando comparado com outras espécies que possuem nessas mesmas condições protocolos estabelecidos.

Nessa mesma espécie a criopreservação com uso do crioprotetor externo trealose, na concentração final de 200Mm demonstrou um resultado próximo ao já descrito em outras espécies de peixes siluriformes. Entretanto, se faz necessário para confirmar esses resultados a realização de estudos *in vivo* de fertilização e eclosão de ovos após o uso dessas amostras na fertilização artificial.

O diluente e o protocolo de congelamento testados no presente estudo fornecem um método promissor, pois esse foi o primeiro passo para se estabelecer um protocolo de criopreservação para a espécie, viabilizando o emprego desta biotecnologia para programas de melhoramento animal em pisciculturas e auxiliando na preservação da diversidade genética. Sendo, uma alternativa para garantir a disponibilidade de gametas em períodos de reprodução, visto a importância que está espécie *L. marmoratus* representa comercialmente.

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Anexos

Projeto Aprovado pelo Cocepe - 6393

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PRPPG – Pró-Reitoria de Pesquisa e Pós-Graduação UNIVERSIDADE FEDERAL DE PELOTAS PPGV – Programa de Pós-Graduação em Veterinária

Projeto de Pesquisa

Área de Conhecimento: Sanidade Animal

Criopreservação e identificação de proteínas do plasma seminal de jundiá amazônico *(Leiarius marmoratus*).

Equipe:

1. ***Pesquisadores***

Antonio Sergio Varela Junior, Doutor em Aquicultura;
Carine Dahl Corcini, Doutora em Biotecnologia;
Thomaz Lucia Jr., Doutor em Medicina Veterinária.

2. ***Alunos de Pós-Graduação***

Alessandra Cardoso da Silva, Mestranda em Veterinária, UFPel
Sara Lorandi Soares, Doutoranda em Biotecnologia, UFPel
Stela Mari Meneghello Gheller, Doutoranda em Veterinária, UFPel

3. ***Alunos de Graduação***

Francisco de Assis Araújo Camelo, Graduando em Medicina Veterinária, UFPel.
Edenara Anastácio ds Silva, Graduanda em Medicina Veterinária, UFPel.
Geórgia da Cruz Tavares, Graduanda em Medicina Veterinária, UFPel.
Rutiele Nolasco Rickes, Graduando em Zootecnia, UFPel