UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE AGRONOMIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA

IURI MORAES NEYRÃO

CRIOPRESERVAÇÃO EM SÊMEN DE PEIXES: REVISÃO DE PROTOCOLOS EXPERIMENTAIS E NOVAS PERCEPÇÕES

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CRIOPRESERVAÇÃO EM SÊMEN DE PEIXES: REVISÃO DE PROTOCOLOS EXPERIMENTAIS E NOVAS PERCEPÇÕES

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Mestre em Aquicultura

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“A ciência nunca resolve um problema sem criar pelo menos outros dez”.

George Bernard Shaw (1856-1950)

CRIOPRESERVAÇÃO EM SÊMEN DE PEIXES: REVISÃO DE PROTOCOLOS EXPERIMENTAIS E NOVAS PERCEPÇÕES[1]

Autor: Iuri Moraes Neyrão
Orientadora: Vivian Fisher
Coorientador: Leandro Cesar de Godoy

Resumo: A criopreservação de espermatozoides tem sido alcançada com sucesso para muitas espécies aquáticas, especialmente em peixes. A utilização do leite em pó tem se mostrado uma alternativa interessante para criopreservação espermática, e seu uso tem apresentado bons resultados após o descongelamento para diversas espécies de peixe. O zebrafish é um modelo biológico popular para estudos de diversas áreas de pesquisa, apesentando particularidades satisfatórias, por isso se faz necessária a preservação das linhagens existentes. Essa tese teve como objetivo geral realizar um levantamento do histórico e do momento recente sobre a criopreservação de sêmen em diversas espécies de peixes, bem como conceituar uma avaliação crítica das limitações, possíveis tendências e caminhos a serem seguidos nesta área, além de realizar a análise do espermatozoide de zebrafish em contato com diferentes crioprotetores e tempos de equilíbrio, tomando como ferramenta de estudo as relações estruturais, morfológicas e de integridade de membrana do espermatozoide. Com os resultados obtidos no primeiro estudo, foi possível obter informações detalhadas sobre protocolos de criopreservação, taxas de resfriamento e métodos de descongelamento, dispositivos utilizados, bem como a associação do leite em pó com outros crioprotetores, além de conceituar uma avaliação crítica das limitações, possíveis tendências e caminhos a serem seguidos na área da criopreservação. A partir das respostas obtidas foi possível observar que a inclusão do leite em pó na crioproteção de espermatozoides trouxe respostas positivas, apresentando ganhos satisfatórios principalmente para espécies pertencentes à ordem Siluriformes e Cypriniformes. No segundo estudo, pôde-se observar que o tratamento com concentração de 10% de metanol e 5 minutos de equilíbrio foi o mais indicado, apresentado melhores resultados para a realização do processo de criopreservação espermática. A avaliação da relação de simultaneidade entre os fatores crioprotetor, concentração do crioprotetor e tempo de equilíbrio ao crioprotetor é de suma importância, pois a resposta é prática e objetiva, resposta essa que aumenta a taxa de sucesso experimental para a criopreservação, além de servir como referência para novos e mais refinados ensaios a serem desenvolvidos na área.

Palavras-chave: Leite em pó; revisão sistemática; espermatozoide; *Danio rerio*; morfologia; integridade de membrana

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[1]Tese de Doutorado em Zootecnia – Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (157 p.) Março, 2022.

CRYOPRESERVATION IN FISH SPERM: REVIEW OF EXPERIMENTAL PROTOCOLS AND NEW PERCEPTIONS [2]

Author: Iuri Moraes Neyrão
Supervisor: Vivian Fisher
Co-supervisor: Leandro Cesar de Godoy

Abstract: Sperm cryopreservation has been successfully achieved for many aquatic species, especially fish. The use of powdered milk has shown to be an interesting alternative for sperm cryopreservation, and its use has shown good results after thawing for several species of fish. Zebrafish is a popular biological model for studies in several areas of research, presenting satisfactory particularities, so it is necessary to preserve the existing lineages. This thesis had as general objective to carry out a survey of the history and recent moment on the cryopreservation of semen in several species of fish, as well as to conceptualize a critical evaluation of the limitations, possible trends and paths to be followed in this area, in addition to performing the analysis of zebrafish sperm in contact with different cryoprotectants and equilibrium times, taking the structural, morphological and membrane integrity relationships of the sperm as a study tool. With the results found in the first study, it was possible to obtain detailed information about cryopreservation protocols, freezing rates and thawing methods, devices used, as well as the association of powdered milk with other cryoprotectants, in addition to conceptualizing a critical assessment of the limitations, possible trends and paths to be followed in the area of cryopreservation. From the results obtained, it was possible to observe that the inclusion of powdered milk in the cryoprotection of spermatozoa brought positive responses, showing satisfactory gains mainly for species belonging to the order Siluriformes and Cypriniformes. In the second study, it was observed that the treatment with a concentration of 10% methanol and 5 minutes of equilibrium was the most indicated, presenting better results for the sperm cryopreservation process. The evaluation of the simultaneity relationship between cryoprotectant factors, cryoprotectant concentration and cryoprotectant equilibrium time is of paramount importance, since the answer is practical and objective, a response that increases the experimental success rate for cryopreservation, in addition to serving as a reference for new and more refined tests to be developed in the area.

Keywords: Powdered milk; systematic review; sperm; *Danio rerio*; morphology; membrane integrity

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LISTA DE ABREVIATURAS E SÍMBOLOS

- - Negative Results
+ - Good Results
= - Equal Results
BSA - Albumina Sérica Bovina
BSMIS - Buffered Sperm Motility-Inhibiting Solution
BSP - Binder of SPerm
CASA - Computer-Assisted Sperm Analysis
cm - Centímetro(s)
CPAs - Cryoprotectants
CRISPR/Cas9 - Clustered Regularly Interspaced Short Palindromic Repeats Associated Protein 9
Da - Dalton (s)
DMA - Dimetilacetamida
DMF – Dimethylformamide/Dimetilformamida
DMSO - Dimethyl Sulfoxide/Dimetilsulfóxido
DNA - Ácido Desoxirribonucleico
E400 - Sperm Extender
GFR - Ginsburg Fish Ringer
GSI - Gonadosomatic Index
HBSS - Hanks' Balanced Salt Solution
HES - Hidroxietilamido
K - Condition Factor
LN - Liquid Nitrogen
M - Molar
MET - Methanol/Metanol
mg – Milligrams/miligrama (s)
ml - Milliliters
mM - Milimolar
mm - Millimeters
mmol - Millimole
NE - Not Have Effect
nm - Nanometer
nM - Nanomole
PI - Propidium Iodide
PVP - N-vinil-2-pirolidona
RMMB - Raffinose Freezing Medium
s - Segundos
SYBR-14 - Fluorescent Nucleic Acid Stain
TILLING - Targeting Induced Local Lesions in Genomes
β-lactoglobulin - Beta-lactoglobulin
µl - Microliter (s)

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CAPÍTULO I

1. INTRODUÇÃO

O desenvolvimento do setor aquícola, ligado à necessidade de conservação de espécies ameaçadas, tem estimulado cada vez mais o interesse na preservação de recursos genéticos de espécies com importância econômica e ecológica (Martínez-Páramo et al., 2017). A técnica de armazenamento a longo prazo em temperaturas baixas (Tsai & Lin, 2012) - se faz necessária, induzindo as células à parada das reações enzimáticas, possibilitando, após o descongelamento, a restauração da atividade metabólica (Mazur et al., 1984).

Diversos protocolos de criopreservação são pesquisados e aprimorados frequentemente (Chen et al., 2010; de Carvalho et al., 2014; Fabbrocini, et al., 2014), todavia o processo de criopreservação gera várias condições que podem levar a danos celulares graves (Carmichael et al., 2009). Estes incluem danos celulares, formação de gelo intracelular e extracelular e desidratação na presença do crioprotetor. Quando os tecidos são resfriados lentamente, a água se move para fora das células e o gelo se forma no espaço extracelular (Meryman, 1956; Gage & Baust, 1998; Robilotto et al., 2018). O movimento da água para fora das células pode levar à desidratação celular, e o excesso de gelo extracelular pode levar a danos mecânicos por esmagamento (Mazur, 2004). Embora alguns organismos e tecidos possam tolerar o gelo extracelular, os cristais de gelo intracelular quase sempre danificam gravemente as células (Pegg, 1987; Fowler & Toner, 2006; Nishiyama et al., 2016). Solutos que são excluídos da estrutura do cristal de gelo durante o congelamento afetarão o equilíbrio da pressão osmótica (Honeyfield & Krise, 2000).

Os riscos de dano celular podem ser bastante reduzidos pela adição de um crioprotetor à solução de congelamento, que reduz a temperatura de congelamento e aumenta a viscosidade do líquido (Cabrita et al., 2003).

Os crioprotetores podem ser divididos em duas categorias: crioprotetores intracelular e extracelular. Os crioprotetores intracelulares, como o dimetilsulfóxido (DMSO), metanol (MET), dimetilacetamida (DMA), dimetilformamida (DMF) e glicerol, geralmente possuem peso molecular menor que 400 Da e podem cruzar passivamente as membranas celulares, enquanto que os crioprotetores extracelulares não são difusíveis e, desta forma, não podem atravessar a membrana celular devido ao seu alto massa molecular; como os constituintes do

leite em pó (Carmichael et al., 2009), o hidroxietilamido (HES) e o poli [N-vinil-2-pirolidona] (PVP) (Karlsson & Toner, 1996).

Substâncias diferentes foram investigadas com relação a eficiência para atuar como crioprotetores, incluindo álcoois, dióis, amidas, açúcares e aminoácidos (Freimark et al., 2011; Sun et al., 2012; Hofmann et al., 2015). No entanto, pouco se sabe quão tóxicos os crioprotetores podem ser para as células espermáticas quando expostas a estes, mesmo que por um curto período de tempo.

Estudos foram realizados avaliando as diferentes soluções de criopreservação sobre parâmetros químicos e bioquímicos da qualidade do sêmen de peixes, integridade de membrana e estresse oxidativo (Li et al., 2010, 2013; Wang et al., 2015; Martínez-Páramo et al., 2017). Os protocolos fazem a inclusão de leite em pó comercial em soluções crioprotetoras, favorecendo inúmeros problemas, dentre eles a dificuldade da avaliação espermática, assim como uma falta de padronização dos ingredientes, conforme observado em nosso laboratório. Por exemplo, existem diferentes fontes de leite em pó, de diferentes origens, inclusive vegetal, que possuem maior suplementação de aminoácidos e vitaminas em sua composição que o leite em pó mais comercialmente consumido, mas que ainda não passaram por processos experimentais.

O zebrafish (*Danio rerio*) é um peixe de água doce cujo tamanho pequeno, fecundidade, transparência embrionária e desenvolvimento rápido levaram a sua seleção como modelo experimental popular e de fácil manejo (Van Eeden et al., 1998; Briggs, 2002). Nos últimos anos, o uso generalizado do zebrafish como espécie modelo para vários campos nas ciências biológicas e similaridade genética com humanos propiciou a geração de indivíduos mutantes, assim como, a produção de linhagens transgênicas (Brand et al, 2002; Bai et al., 2013). Isso levou a necessidade de armazenar materiais genéticos, que uma vez congelados estariam protegidos contra qualquer perda acidental da linhagem do animal, reduzindo assim o espaço necessário de manutenção das linhagens dos peixes que não estão em utilização.

Os protocolos de criopreservação do sêmen de zebrafish atualmente usados pela comunidade científica possuem pouca repetitividade. Aliado à questão, as taxas de fertilização usando sêmen criopreservado variam consideravelmente entre laboratórios, assim como o conhecimento do efeito tóxico da criopreservação com

a combinação de crioprotetores permeáveis e não permeáveis, provavelmente devido aos inúmeros protocolos que foram desenvolvidos de maneiras diferentes. Para o sucesso efetivo do uso desta tecnologia faz-se necessário um estudo aprofundado da relação entre crioprotetor e espermatozoide, avaliando as alterações e possíveis lesões celular ao longo do tempo. A partir deste conhecimento, pode-se então, determinar um protocolo viável de criopreservação de espermatozoides de zebrafish que seja de fácil reprodutibilidade.

2. REVISÃO BIBLIOGRÁFICA

2.1 Zebrafish (*Danio rerio*)

O zebrafish (*Danio rerio*) é um teleósteo tropical de água doce, originário da Ásia, pertencente à família Cyprinidae (Engeszer et al., 2007). A espécie apresenta pequeno tamanho, atingindo em torno de 4-5 cm de comprimento (Spence et al., 2008). Possui hábito alimentar onívoro, não possui estômago verdadeiro e tem longo intestino com uma grande área de absorção (Ulloa et al., 2011). Na natureza, o zebrafish se alimenta de larvas de mosquito e pequenos insetos (Spence et al., 2008).

O zebrafish é encontrado comumente em rios, riachos pequenos e outros canais, em águas estagnadas ou lênticas perto de córregos e arrozais, com larguras dos corpos d'água variando de 1 até 12 m, e profundidades variando de 16 a 57 cm; com características de água relativamente calma, clara, apresentado temperatura de 27 à 34°C e pH entre as faixas de 7,9-8,2; em substratos de argila ou pedras (Engeszer et al., 2007). O *Danio rerio* é encontrado na natureza em cardumes de diferentes tamanhos, em pequenos grupos de 6 ou 7 peixes em águas lentas, cardumes de 22 peixes em águas paradas e chegando até de 300 peixes águas com grande fluxo (Graham et al., 2018).

A espécie possui dimorfismo sexual, com as diferenças na coloração iniciando após seis semanas de vida (Darrow & Haris, 2004), principalmente na fase reprodutiva. Ao amanhecer os machos possuem coloração nitidamente amarelo/dourado na parte ventral, e as fêmeas apresentam cor prateada (Paull et al., 2010). Na fase reprodutiva os machos apresentam padrão corporal mais afilado que as fêmeas e são mais leves, no entanto, apresentam o mesmo comprimento (Eaton & Farley, 1974; Lawrence, 2007).

O *Danio rerio* é um peixe ovulíparo, ou seja, sua fertilização e o desenvolvimento ocorrem no meio externo. O zebrafish possui condições de se reproduzir dos quatro meses de vida até os 3 anos, todavia, a capacidade reprodutiva diminui com o avanço etário, e a recomendação de vários laboratórios é que a reprodução seja realizada com animais entre 6 até 18 meses de idade (Nasiadka & Clark, 2012; Nishimura et al., 2016). O testículo do zebrafish é

classificado como do tipo tubular anastomasado (Maack & Segner, 2003; Fenske & Segner, 2004; Nash et al., 2004).

2.1.1 Importância atual do zebrafish nas áreas do conhecimento

O *Danio rerio* apresenta características favoráveis à escolha como modelo experimental, como a alta fecundidade, tamanho diminuto, rápido tempo entre gerações, transparência óptica durante a embriogênese e homologia genética com humanos (Lawrence, 2007; Spence et al., 2008); custos de manejo e manutenção são inferiores aos de outros animais utilizados na pesquisa, como os camundongos. Com a grande homologia de seu genoma com o de outras espécies de vertebrados, em especial os humanos, que apresentam cerca de 70% dos genes em comum com o zebrafish (Howe et al., 2013; Johnston et al., 2018), estudos com a espécie avançaram e se tornaram um organismo popular no estudo de genética, biologia do desenvolvimento e biomedicina (Kalueff et al., 2014; Fontana et al., 2018). A espécie vem sendo utilizada como modelo em pesquisas, como: testes de segurança com fármacos, avaliação de riscos ambientais e toxicidade durante o desenvolvimento (Tanoue et al., 2015; Kristofco et al., 2015).

Existe interesse crescente na utilização do zebrafish como organismo modelo para pesquisas comportamentais, pois seu comportamento é influenciado por manipulações ambientais e farmacológicas, podendo ser estudado baseado no comportamento exploratório, e também em respostas do sistema endócrino ao estresse (Egan et al., 2009; Bengston et al., 2018).

2.2. Criopreservação: Uso do zebrafish na criobiologia

A criopreservação é uma biotecnologia que vem sendo bastante utilizada na preservação do material genético de peixes, sendo de suma importância na preservação da diversidade de espécies e modelos de peixes utilizados em pesquisas biomédicas (Lin et al., 2016). A criopreservação é normalmente realizada objetivando a estagnação do metabolismo, das atividades biológicas e reações bioquímicas que causariam envelhecimento e morte celular (Mazur et al., 1984; Sieme et al., 2015). Para uma taxa de resfriamento ser considerada ideal, a mesma necessita ocorrer o mais rápido possível, para se evitar os efeitos tóxicos da

solução crioprotetora, no entanto, lento o suficiente para que as células possam desidratar o suficiente para evitar a formação de gelo intracelular (Mazur et al., 1963, 1984; Jin et al., 2016).

O zebrafish possui grande participação em estudos relacionados à criopreservação, apresentando técnicas úteis e eficientes para a preservação de valiosas linhas de pesquisa. Aliado a pesquisa, as relações processuais das atividades de criopreservação envolvem desenvolvimento inicial de protocolos de criopreservação, aplicação dos protocolos e aplicação em grande escala em recursos genéticos e indústria comercial (Yang & Tiersch, 2009; Yang et al., 2016).

2.3. Criopreservação de sêmen

2.3.1 Comparativo entre espécies de peixes

Desde as primeiras tentativas de criopreservar espermatozoides de peixes, a potencial aplicação metodológica para espécies de água doce e marinhas tem atraído a atenção dos pesquisadores. Protocolos de congelamento foram desenvolvidos para diferentes espécies em todo o mundo e diversos estudos têm se aprofundado em espécies de climas temperados com período reprodutivo de forma sazonal.

O primeiro protocolo de criopreservação para *Danio rerio* relacionado ao sêmen foi publicado em 1982 por Harvey et al. (1982), tendo embasado toda comunidade científica da área na busca de novos protocolos (Morris et al., 2003; Yang et al., 2007; Carmichael et al., 2009; Draper & Moens, 2009; Bai et al., 2013; Wang et al., 2015; Yang et al., 2016; Matthews et al., 2018; Rodrigues et al., 2020, 2021). Os protocolos desenvolvidos até o momento variam desde o método de coleta do sêmen, a combinação de diferentes diluentes e crioprotetores, no recipiente utilizado para o congelamento, no método de congelamento e curva de resfriamento, produzindo, desta maneira, resultados variáveis (Tiersch, 2001; Hagedorn & Carter, 2011), conforme apresentado na Tabela 1.

Tabela 1. Compilado de protocolos de criopreservação de sêmen de zebrafish (*Danio rerio*)

Fator	Harvey et al., 1982	Morris et al., 2003	Yang et al, 2007	Draper & Moens, 2009	Carmichael et al., 2009	Bai et al., 2013	Wang et al., 2015;	Yang et al., 2016	Matthews et al., 2018	Rodrigues et al., 2020	Rodrigues et al., 2021
Crioprotetor	Metanol 10%	DMA 10%	MET 8%	MET 8%	MET 10%	DMSO 8%	DMSO 8%	MET 8%	RMMB	MET 8%	MET 8%
Diluidor	GFR	BSMIS	HBSS	GFR	GFR	HBSS	HBSS	HBSS	HBSS	HBSS	HBSS
Coleta	Extrusão	Remoção cirúrgica	Remoção cirúrgica	Extrusão	Extrusão	Remoção cirúrgica	Remoção cirúrgica	Remoção cirúrgica	Extrusão	Remoção cirúrgica	Remoção cirúrgica
Armazenamento	Capilar	Capilar	Palheta	Criotubo	Criotubo	Palheta	Palheta	Palheta	Criotubo	Criotubo	Criotubo
Diluição	HBSS	BSMIS	HBSS	HBSS	HBSS	HBSS	HBSS	HBSS	E400	HBSS	HBSS
Método de congelamento	Gelo seco	Gelo seco	Congelador programável	Gelo seco	Gelo seco	Congelador programável	Congelador programável	Congelador programável	Gelo seco	Gelo seco	Gelo seco
Tempo de resfriamento (min)	30	30	-	20	20	-	-	-	20-60	20	20
Taxa de resfriamento	16 °C/min	?	10 °C/min	?	30 °C/min (1-2 min) e 2–3 °C/min (3-10 min)	25 °C/min (4 a -30°C), e 5 °C/min (-30 a -80°C)	25 °C/min (4 a -30 °C, e 5 °C/min (-30 a -80 °C)	10 °C/min (4 a -80 °C)	16,1 °C/min	14-16ºC/min	14-16ºC/min
Taxa de Motilidade (%)	43 ± 12	12 ± 6	78 ± 10	?	?	46,8 ± 6,40	28 ± 4	28 ± 15	82 ± 2	47,6 ± 10,4	-
Descongelamento	Ambiente	37 °C	40,5 °C, 5 s	33 °C, 8 s	33 °C	40 °C, 7 s	40 °C, 7 s	40°C, 5 s	38ºC, 10-15 s	38 °C, 10 s	38 °C, 10 s
Taxa de Fertilização (%)	51 ± 36	14 ± 10	33 ± 20	28 ± 18	?	-	62 ± 6	73 ± 21	33 ± 19	-	-

Legendas: Ginsburg Fish Ringer (GFR); BSMIS (75mM NaCl, 70mM KCl, 2mM $CaCl_2$, 1mM MgSO4, e 20mM Tris); Hanks' Balanced Salt Solution (HBSS); Metanol - CH_3OH (MET); Dimetilsulfóxido - $C2H_6OS$ (DMSO); Dimetilacetamida - C_4H_9NO (DMA); RMMB (20% Raffinose penta hidratada, 2,5% Leite em pó desnatado, 6,67% Metanol, 30mM tampão de Bicina); E400 (130mM KCL, 50mM NaCl, 2mM $CaCl_2$, 1mM $MgSO_4$, 10mM Glicose e 30mM HEPES-KOH)

A maioria dos estudos recentes, os quais apresentam análises mais refinadas, é aplicada para salmonídeos, ciprinídeos e esturjões. Todavia, apesar de ainda pequeno, o interesse em realizar protocolos de criopreservação para espécies de áreas tropicais e subtropicais é crescente (Maria et al., 2006, 2015; Viveiros & Godinho, 2009; Pastana et al., 2019; Neumann et al., 2019). Diversos experimentos foram realizados para avaliar uma série de fatores como taxas de congelamento, crioprotetores e soluções diluidoras com o objetivo de desenvolver protocolos de criopreservação para espécies como *Salmo salar L.* (Dziewulska et al., 2011), *Huso huso* (Aramli et al., 2015), *Acipenser baerii* (Judycka et al., 2015), *Perca fluviatilis* (Bernáth et al., 2015; Judycka et al., 2019) e C*olossoma macropomum* (Maria et al., 2015; Varela Junior et al*.*, 2015). A avaliação da qualidade do sêmen de peixes após o congelamento/descongelamento evoluiu, permitindo melhor compreensão dos mecanismos ligados a qualidade que era comprometida durante o processo de criopreservação.

A análise de motilidade é comumente realizada usando sistemas informatizados (Martínez-Páramo et al., 2017). O uso da citometria de fluxo melhorou a confiabilidade dos resultados sobre a qualidade da membrana e estado mitocondrial (Arruda et al., 2007). O estresse oxidativo é avaliado utilizando diferentes metodologias, assim como a integridade da cromatina, sendo este, também considerado como uma avaliação importante (Cabrita et al., 2014; Fernández et al., 2019). Outras recentes tecnologias têm colaborado para a melhor compreensão da fisiologia dos espermatozoides, como o uso de análises proteômicas, que revelam mudanças nas proteínas relacionadas ao tráfico e organização da membrana, metabolismo ou transdução de sinal (Li et al., 2010, 2013; Nynca et al., 2015a). Além da taxa de fertilização, diferentes estudos avaliaram o desenvolvimento, a longo prazo, da progênie (Pérez-Cerezales et al., 2011; Viveiros et al., 2012).

A redução do estresse oxidativo não foi alcançado pela adição de diferentes compostos em soluções diluidoras antes do congelamento. Existe um número de compostos naturais e antioxidantes sintéticos que apresentavam diferentes níveis de eficiência, dependendo da espécie, concentração ou se forem combinados com outros reagentes (Martínez-Páramo et al., 2017). Lahnsteiner et al. (2011), testaram 10 soluções antioxidantes contendo catalase, superóxido dismutase, peroxidase,

glutationa reduzida, metionina reduzida, glutationa oxidase e metionina oxidase mas nenhuma das soluções mostrou melhora significativa na qualidade do espermatozoide pós-descongelamento em *Salvelinus fontinalis* e *Oncorhynchus mykiss*.

Progressos no congelamento de sêmen em carpas incluem diferentes modificações, técnicas e da adição de vários compostos para o meio de congelamento, a fim de aumentar a crio-resistência. Nestas espécies, a proteção da membrana foi aumentada utilizando 1,5 mg por 120 x 10^6 espermatozoides de ciclodextrina carregada de colesterol, que resultou em aumento significativo na motilidade, viabilidade e fertilidade (Yildiz et al., 2015; Partyka et al., 2018). A adição de 20 mM de cisteína proporcionou maior motilidade, taxas de fertilidade e eclosão e menor nível de danos no DNA (Öğretmen et al., 2015; Li et al., 2018).

Para salmonídeos, taxas de fertilidade semelhantes as obtidas com espermatozoides controle foram alcançadas em relação aos espermatozoides e oócitos, obtendo os valores 50.000:1 para *Thymallus thymallus* (Horváth et al., 2015), 300.000:1 para *S. fontinalis* e *Salmo trutta* (Nynca et al., 2014, 2015b), 500.000:1 para *Hucho hucho* L. (Nynca et al., 2015c) e 600.000:1 para *O. mykiss* (Ciereszko *et al.*, 2014). A vitrificação é uma técnica raramente usada para sêmen de peixe, todavia possui aplicações promissoras. A vitrificação de espermatozoides diluídos em meio de Cortland com 10% de DMSO, 2% de BSA, 0,13 M sacarose e 50% de plasma seminal foram relatados como uma opção na criopreservação para *S. salar* L. (Figueroa et al., 2015). Os autores relataram boas condições para integridade da membrana, motilidade e fertilidade e baixas taxas de fragmentação do DNA nessas condições.

2.3.2 Principais dificuldades encontradas

Nos últimos anos, ensaios relacionados à genética geraram milhares de novas variedades de zebrafish (Driever et al., 1996; Haffter et al., 1996; Kettleborough et al., 2013; Thyme et al., 2019). Além disso, várias novas tecnologias tornaram-se disponíveis, como geração de linhas repórter transgênicas, *knockout* dirigido de genes (Meng et al., 2008; Doyon et al., 2008; Kok et al., 2015; Adamson et al., 2018), TILLING (Targeting Induced Local Lesions In Genomes) (Draper et al., 2004; Amsterdam & Hopkins, 2006; Kidwell et al.,

2018), inserções baseadas em vírus e *transposon* (Driever et al., 1996; Haffter et al., 1996; Amsterdam et al., 1999; Lieschke & Currie, 2007; Donnarumma, et al., 2018) e potencializadoras ou estratégicas de armadilha genética (Kawakami, 2005, 2007; White, et al., 2019). Vários programas de mutagênese usarão essas novas tecnologias para gerar, identificar e caracterizar milhares de genes em um futuro próximo (http://www.zf-models.org/). Assim, o aumento adicional no número e variedade de linhagens de *Danio rerio* é assertivo.

Apesar do custo significativo para gerar essas linhagens, pouco esforço tem sido feito para preservá-las com segurança para futuras gerações de pesquisadores. Normalmente, a capacidade dos laboratórios em manter os animais é limitada pelo número de pessoas e espaço reduzido das instalações (Carmichael et al., 2009). Muitas linhagens correm o risco de se extinguirem sem um esforço para financiá-las.

Atualmente, a criopreservação é o método mais eficiente para preservar amostras de espécies ameaçadas e para armazenar materiais genéticos raros ou importantes para manutenção da diversidade genética. A criopreservação é a melhor alternativa para os estoques vivos por vários motivos: 1) O espaço da instalação é eficientemente utilizado; 2) O tempo reprodutivo efetivo dos machos é prolongado; 3) Recursos importantes não se extinguem se uma linha não puder ser mantida viva; 4) mutagêneses genéticas reversas, como TILLING, são mais viáveis pois os peixes vivos não precisam ser mantidos (Draper et al., 2004; Amsterdam & Hopkins, 2006; Kidwell et al., 2018); 5) A criopreservação é custo-efetiva, devido a economia de recursos necessários para eletricidade, água, filtragem, limpeza de tanques, reparos, alimentos e pessoal.

No entanto, os protocolos de criopreservação atualmente usados pela comunidade científica para zebrafish podem ser relativamente difíceis de conduzir e os laboratórios não podem adaptá-los facilmente. Além disso, as taxas de fertilização variam consideravelmente entre laboratórios, provavelmente devido aos inúmeros protocolos diferentes que foram desenvolvidos (Morris et al., 2003; Sood et al., 2006; Jing et al., 2009; Marinović et al., 2018) como modificações do procedimento original por Walker e Streisinger (Westerfield, 2007), que foi baseado no método original de criopreservação de Harvey et al. (1982). Essas modificações foram amplamente testadas empiricamente, determinando se elas afetam ou não

as taxas gerais de fertilização pós-descongelamento (Carmichael et al., 2009). Sem um profundo entendimento dos princípios biofísicos que governam a fisiologia das células congeladas, não está claro se a comunidade atualmente usa o método mais prático, confiável e eficiente.

Os riscos de dano celular podem ser muitos reduzidos pela adição de um crioprotetor à solução de congelamento, a qual reduz o ponto de congelamento e aumenta a viscosidade do líquido; dessa forma, ao invés de cristalizar, a solução forma gelo amorfo (vitrificação). A vitrificação da água também pode ser obtida através de um resfriamento extremamente rápido, na ausência de um crioprotetor (Isachenko et al., 2005; Ozkavukcu et al., 2008; Hezavehei et al., 2018). Para conseguir esses efeitos dentro da célula, os crioprotetores adentram rapidamente na membrana celular. No entanto, os crioprotetores comuns tais como metanol, glicerol, DMSO ou DMA em vários momentos são tóxicos em altas concentrações. Portanto, um dos desafios para desenvolver um método eficaz de criopreservação é limitar o dano produzido pelo próprio crioprotetor (Carmichael et al., 2009).

2.4. Avaliação da qualidade do sêmen criopreservado

A análise de motilidade espermática é bastante útil para a comparação de diferentes condições experimentais, como procedimentos de coleta, meios de diluição e armazenamento de sêmen (Bobe & Labbé, 2010), em especial na criopreservação. A motilidade já possui avaliação de 340 espécies de peixes (Gallego & Asturiano, 2019), sendo uma excelente análise para a realização após os processos de criopreservação.

O estudo morfológico das células espermáticas e sua relação com a infertilidade dos machos alcançaram maior importância através da inseminação artificial em mamíferos, especialmente na espécie bovina (Blom, 1973; Kavamoto et al., 2018). A análise da morfologia espermática tem por sua função um importante fator na avaliação da qualidade seminal, pois que o aumento das patologias espermáticas provoca diminuição na motilidade e no vigor espermático (Lahnsteiner et al., 1998; Cosson et al., 1999; Streit Junior et al., 2018).

A avaliação de integridade de membrana auxilia no sucesso da criopreservação, uma vez que as membranas são suscetíveis às criolesões (Jun et al., 2006; Kutluyer et al., 2016; Xin et al., 2018). A integridade da membrana é

geralmente avaliada com a utilização de corantes como eosina/nigrosina ou eosina isolada e uma observação simples sob microscópio e utilizando kits com marcadores específicos (Sybr-14/iodeto de propídio), permite uma observação simultânea de espermatozoides vivos ou mortos (Fauvel et al., 2010; Gallego et al., 2013; Gallego & Asturiano, 2019). No entanto, diversas outras técnicas baseadas no uso de outros corantes são encontradas na literatura, como o iodeto de propídio (Fauvel et al., 2010) e azul de tripan (Lubzens et al., 1997).

3. HIPÓTESES E OBJETIVOS

3.1. Hipóteses

As hipóteses propostas pelo presente estudo seriam que (i) o leite em pó, adicionado à solução crioprotetora, atua de maneira eficiente na proteção dos espermatozoides de peixes. Além disso, acredita-se que (ii) o equilíbrio em determinadas concentrações e tempos de equilíbrio possa levar a alterações na qualidade dos espermatozoides de zebrafish após o descongelamento das amostras, principalmente danos morfológicos, estruturais e de integridade de membrana.

3.2. Objetivos

3.2.1. Objetivo Geral

- Fornecer, por meio da revisão sistemática, uma avaliação da história e cenário atual da criopreservação de sêmen em diversas espécies de peixes e avaliar e compreender as relações estruturais e de viabilidade do sêmen de zebrafish (*Danio rerio*) em contato com diferentes crioprotetores e tempos de equilíbrio.

3.2.2. Objetivos Específicos

- Revisar, de forma sistemática, a compilação de dados de artigos científicos que realizaram a utilização de leite em pó em sua composição para criopreservação de sêmen em diferentes espécies de peixes;
- Reunir informações detalhadas sobre protocolos de criopreservação, taxas de resfriamento e métodos de descongelamento, dispositivos utilizados, bem como a associação do leite em pó com outros crioprotetores;
- Avaliar as principais análises de viabilidade realizadas após a criopreservação, o efeito do leite em pó através da ação de suas diversas substâncias ao mesmo tempo e se de fato foram obtidos melhores resultados ao adicionar leite em pó na criosolução;

- Analisar a relação estrutural e morfológica do espermatozoide pós-criopreservação para zebrafish;
- Avaliar a viabilidade do sêmen de zebrafish submetido a diferentes soluções crioprotetoras e tempos de equilíbrio.

CAPÍTULO IV

4. CONSIDERAÇÕES FINAIS

Pode-se avaliar que o leite em pó como um complexo de várias substâncias importantes que agem de forma diferente e sincronizada, atuando na proteção do espermatozoide quando combinado com soluções protetoras das mais diversas concentrações, contra efeitos de radicais livres e exposição a baixas temperaturas.

Apesar dos resultados benéficos, ressalta-se que as diferenças na composição e qualidade das diferentes marcas de leite em pó disponíveis mundialmente possam ser um fator limitante para a padronização de protocolos de criopreservação com este composto, e com isso, o estabelecimento de uma composição básica mínima de um produto e a padronização deve ser priorizada no futuro, com o objetivo de realizar a aplicação dos protocolos nos mais diferentes laboratórios do mundo. E conforme foi observado, as evidências científicas apresentadas podem contribuir e servir de ponto de partida para novos e mais refinados estudos a serem desenvolvidos na área.

A utilização de diferentes crioprotetores, concentrações de crioprotetores e tempos de equilíbrio são encontrados em diversos protocolos de criopreservação voltados para o sêmen de zebrafish, porém considerando cada variável de forma isolada, sem uma relação de simultaneidade entre os fatores, principalmente levando em consideração o tempo de equilíbrio do crioprotetor em contato com o material biológico.

Dessa forma, encorajamos que mais testes possam ser feitos com o metanol em diferentes concentrações, diferentes tempos de equilíbrio, mas também verificando a atuação e desempenho de outros crioprotetores, favorecendo a busca de protocolos que possam reduzir a toxicidade do meio crioprotetor, além da busca de protocolos que possam ser cada vez mais práticos, objetivos e aplicáveis nos mais diversos laboratórios. Ao final, se espera que os resultados obtidos posam contribuir e servir como referência para o desenvolvimento do aprimoramento de mais protocolos para crioproteção de sêmen de zebrafish.

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APÊNDICES

Apêndice 1 – Carta de Aprovação Comissão De Ética No Uso De Animais

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA
Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 35329
Título: ESTUDO DO COMPORTAMENTO DE ZEBRAFISH (Danio rerio) PROVENIENTE DE SÊMEN CRIOPRESERVADO

Vigência: 01/06/2018 à 31/12/2020

Pesquisadores:

Equipe UFRGS:

DANILO PEDRO STREIT JR - coordenador desde 01/06/2018
BRUNA BITENCOURT DA COSTA - Aluno de Doutorado desde 01/06/2018
Ana Amélia Nunes Fossati - Aluno de Doutorado desde 01/06/2018
Paula Graziela Lassen - Aluno de Doutorado desde 01/06/2018
Rômulo Batista Rodrigues - Aluno de Doutorado desde 01/06/2018
Bruno da Silva Pires - Aluno de Mestrado desde 01/06/2018

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 17/09/2018 - Sala 330 do Anexo I do Prédio da Reitoria - Campus Centro - Av. Paulo Gama,100/ Porto Alegre - RS, em seus aspectos éticos e metodológicos, para a utilização de 545 zebra fish, oriundos de compra e de fertilização; de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Sexta-Feira, 28 de Setembro de 2018

ALEXANDRE TAVARES DUARTE DE OLIVEIRA
Vice Coordenador da comissão de ética

Apêndice 2 – Normas do periódico Biopreservation and Biobanking

Manuscript Submission Guidelines and Policies for Biopreservation and Biobanking

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Journal Information

- Manuscript Submission Site: https://mc.manuscriptcentral.com/biopreservbiobank
- Editorial Office Contact: ghewitt@liebertpub.com
- Support Contact: prosupport@liebertpub.com
- Journal Model: Hybrid (Open Access Option)
- Blinding: Single Blind
- File formatting requirement stage: Upon submission
- Instant Online Option (immediate publication of accepted version): No
- Submission Fee: None
- Average time to initial decision: 29 days

About the Journal

Biopreservation and Biobanking is the first journal to provide a unifying forum for researchers from academia, government, and industry in which rapid, authoritative, peer-reviewed communication of recent advances is provided in the rapidly emerging field of biospecimen procurement, processing, preservation, and banking. The Journal publishes a diversity of articles focusing on current issues and problems related to the processing of macromolecules, cells, and tissues.
A wide range of topics related to the fundamentals of the basic biopreservation sciences, biosafety, and the ethical, legal and societal issues surrounding biobanking and biorepository operation will be addressed in the Journal.

Manuscript Types and Guidelines

Original Articles	• 3,000-word limit • Structured abstract of no more than 300 words • Maximum total of eight (8) figures and/or tables
Review Articles	• Review articles are summaries of developments in the field. • 5,000-word limit • Structured abstract of no more than 300 words • Maximum total of ten (10) figures and/or tables
Brief Reports and Case Studies	• 1,000-word limit • No abstract is required • Minimum of one (1) figure or table • Maximum of 10 references
Editorials and Commentaries	• Editorials and commentaries may be on any issue relevant to the field, but must be brief and appropriately documented by data. • 1,000-word limit • No abstract • No figures or tables • Maximum of 10 references
Letters to the Editor	• 500-word limit • May include one figure OR table

	• Reference citations are identical in style to those of full original articles, but should not exceed five (5)

Word limits do NOT pertain to the abstract, disclosure statements, author contribution statements, funding information, acknowledgments, tables, figure legends, or references

References

The references should be cited in the text using the numerical order of citation method. The reference list at the end of the paper should be double-spaced and organized numerically by order of citation. List only the first three authors followed by "et al." For accepted papers not yet published, cite the journal and add "in press." If references to personal communications or unpublished data are used, cite them parenthetically in the text, not in the references. Sources for personal communications must provide written approval at the time of submission. References should be presented in Vancouver style as shown below:

Journal example
Ellis, P., Muller-Schweinitzer Maintenance of functional activity of human pulmonary arteries after cryopreservation Br J Pharmacol 1991;103:1377-1380.

Book example:
Toner, M. Nucleation of ice crystals in biological cells. In: Steponkus PL, ed Advances in Low Temperature Biology. London JAI Press; 199 1-52.

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General Manuscript Submission Guidelines and Policies for Mary Ann Liebert Journals

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All manuscripts must be prepared in accordance with the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (icmje.org). Please consult your specific journal's requirements for additional information.
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Apêndice 3 – Normas do periódico Cryobiology

Guide for Authors

Introduction

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Types of article

•Regular Papers
•Brief Communications
•Reviews •Letters to the Editor
Regular papers will describe experimental findings, techniques, or theory. They will consist of an abstract that summarizes the objective of the study, the methods used, and the conclusions reached. Abstracts should not exceed 250 words and should be adequate for direct presentation to abstracting services. After the abstract a list of up to 10 keywords that will be useful for indexing or searching must be included. The Introduction will contain a statement of the purpose of the work, the problem that stimulated it, and a brief summary of relevant published investigations. The Materials and Methods section must be presented in sufficient detail to enable other investigators to repeat the work. The Results should be concise and should avoid redundant tables and figures illustrating the same data. The Discussion should interpret the results, with minimal recapitulation of findings.
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VITA

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Concluiu o ensino fundamental no Centro Nipônico Adventista em 2006 e o ensino médio no Sistema de Ensino Universo em 2009, ambos em Belém-PA. Em 2010 ingressou no curso de Zootecnia, na Universidade Federal do Pampa (UNIPAMPA), campus Dom Pedrito-RS, concluindo sua graduação em 2015/1. Durante a graduação, realizou estágio de iniciação científica no Laboratório de Piscicultura, fazendo parte do grupo de pesquisa NAQUA (Núcleo em Aquacultura). Ao final do curso de graduação, realizou o Estágio Curricular Obrigatório no Laboratório de Aquicultura Continental (LAC), da Universidade Federal do Rio Grande (FURG), onde desenvolveu atividades de pesquisa: nos laboratórios da instituição, manejo dos animais, fabricação de ração e em participação de experimentos de mestrandos e doutorandos que desenvolviam suas atividades de pesquisa.

Em 2015 ingressou no mestrado do Programa de Pós-Graduação em Aquicultura, pela Universidade Estadual Paulista (UNESP), Campus Jaboticabal, sendo Bolsista CNPq. Durante esse período realizou atividades de pesquisa voltadas para organismos aquáticos nas áreas de: fisiologia endócrina e metabólica, bioquímica, imunologia, comportamento e bem-estar. Em 2017, obteve o título de Mestre em Aquicultura, na área de concentração em Aquicultura em Águas Continentais.

Em 2018 ingressou no doutorado do Programa de Pós-Graduação em Zootecnia, pela Universidade Federal do Rio Grande do Sul (UFRGS), na área de concentração de Produção Animal, sendo bolsista CAPES. Nesse período realizou atividades de pesquisa no Laboratório de Piscicultura, além de participar de projetos e reuniões do Projeto ReefBank (Pesquisa Científica e Educação Ambiental para Conservação dos Recifes de Coral), com foco principal no desenvolvimento e aprimoramentos de técnicas relacionadas as metodologias de criopreservação de gametas de organismos aquáticos, além de trabalhos com reprodução e avaliação de qualidade de gametas.